In vitro regeneration of *Bienertia sinuspersici*

by

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Abstract

This thesis presents a study on the development of plant regeneration protocols for *Bienertia sinuspersici,* one of three species of the family Chenopodiaceae that were discovered to perform C_4 photosynthesis in individual chlorenchyma cells. Protocols for *in vitro* regeneration of *B. sinuspersici* were developed as follows: 1) vegetative propagation via adventitious root induction of cuttings was optimized; 2) direct organogenesis and plant regeneration using bud cultures was achieved; 3) plant regeneration via indirect organogenesis of stem-derived callus was established.

All plant materials were obtained from mature, greenhouse grown *B. sinuspersici* plants and cultured *in vitro* on Murashige and Skoog (MS) basal media supplemented with phytohormones. Vegetative propagation by adventitious root formation of cuttings was examined using various strengths of MS medium as well as the effects of auxins [2,4-dichlorophenoxyacetic acid (2,4-D), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), and α-napthalene acetic acid (NAA)] on root development. Half-strength MS medium and IBA were most effective for adventitious root induction. Histological analysis of root anatomy demonstrated the various effects of individual auxin on root development.

Direct organogenesis via bud culture for shoot multiplication and plant regeneration was established. Shoot multiplication occurred on medium supplemented with a cytokinin, 6-benzylaminopurine (BAP). Elongation of multiplied shoots was achieved using medium containing a lower concentration of BAP or gibberellic acid. Rooting of elongated shoots was attempted using IBA *in vitro* or *ex vitro*, and through

micrografting. Rooted shoots were transplanted to soil, acclimated in the greenhouse, and transferred to a growth chamber.

Plant regeneration via indirect organogenesis was also established. Stem explants were cultured on medium containing kinetin and 2,4-D to induce callus formation. Shoot organogenesis from callus tissue was obtained on medium containing thidiazuron (TDZ). *In vitro*-derived shoots were transferred to medium containing BAP for shoot multiplication, and subsequently elongated and rooted as described above. Histological analysis of leaves of regenerated plants from both direct and indirect organogenesis methods showed chlorenchyma cells have the distinctive feature, intracellular compartmentalization of organelles, of the single-cell C_4 system. Immunoblot analysis of proteins isolated from leaves of regenerated plants detected key C_4 photosynthetic enzymes, providing further evidence that single-cell C_4 photosynthesis is present. Cytological analysis of root tips from *in vitro*-derived plants via indirect organogenesis verified normal diploid chromosome number (2n=18) in dividing cells, suggesting neither polyploidy nor aneuploidy occurred in regenerated plants.

Reproducible methods of *in vitro* regeneration of *Bienertia sinuspersici* have been established for vegetative propagation from cuttings, direct organogenesis via axillary bud culture, and indirect organogenesis. These *in vitro* techniques would serve as useful tools for multiplication and genetic transformation of this plant species.

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List of Abbreviations

Photosynthesis

CAM: crassulacean acid metabolism

CCC: central cytoplasmic compartment

G3P: glyceraldehydes-3-phosphate

NAD-ME: nicotinamide adenine dinucleotide malic enzyme

PCC: peripheral cytoplasmic compartment

PPDK: pyruvate-phosphate dikinase

PEPC: phosphenol pyruvate carboxylase

Rubisco: ribulose 1,5 bisphosphate carboxylase/oxygenase

RuBP: ribulose 1,5 bisphosphate

Hormones

Auxins:

2,4-D: 2,4-dichlorophenoxyacetic acid

IAA: indole-3-acetic acid

IBA: indole-3-butyric acid

NAA: α-napthalene acetic acid

Cytokinins

BAP: benzylaminopurine

TDZ: thidiazuron

Other

GA_3: gibberellic acid

Materials

EDTA: ethylenediaminetetraacetic acid

EtOH: ethanol

DTT: dithiothreitol

HCl: hydrochloric acid

LR white: London Resin White

MES: 2-(N-Morpholino)ethanesulfonic acid sodium salt, Monohydrate

MS: Murashige and Skoog (basal media)

NaCl: sodium chloride

SDS: sodium dodecyl sulfate

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

TBO: toluidine blue

TBS-T: Tris-Buffered Saline Tween-20

Tris: tris(hydroxymethyl)aminomethane

PIPES: piperazine-N,N′-bis(2-ethanesulfonic acid (buffer)

PMSF: phenylmethanesulfonylfluoride

PVDF: polyvinylidene difluoride

Miscellaneous

EMS: Electron Microscopy Sciences (supplier)

CIM: callus induction medium

PEG: polyethylene glycol (mediated transformation)

SIM: shoot induction medium

SPSS/ PASW: Statistical Package for Social Sciences/ Predictive Analysis Software

Chapter 1

Introduction

1.1 General Introduction

Tissue culture involves the induction and growth of new plant tissues through the manipulation of *in vitro* culturing conditions. *Bienertia sinuspersici* is one of three species (along with *Bienertia cycloptera* and *Suaeda aralocaspica*) recently discovered to have a novel form of single-cell C_4 photosynthesis in which individual chlorenchyma cells exhibit intracellular compartmentalization of organelles and photosynthetic enzymes to function in a way that is similar to the dual-cell Kranz system. *B. sinuspersici* serves as an example of how plants are able to evolve novel photosynthetic mechanisms to adapt to environmental changes. The evolutionary advantage demonstrated by single-cell C_4 photosynthesis, accomplished by lowering the oxygen environment in one compartment to improve photosynthetic efficiency, makes *B. sinuspersici* a fascinating model system to study. Single-cell C_4 photosynthesis provides an advantage by increasing the efficiency of carbon dioxide assimilation and decreasing photosynthetic product lost to photorespiration without requiring the formation of Kranz anatomy.

The concept of the single-cell C_4 system has offered new prospects to engineer C_4 photosynthesis in C_3 crop plants. To produce transgenic plants, transformation practices require a plant regeneration system to facilitate an efficient means of gene transfer, selection and whole-plant regeneration. *In vitro* techniques provide useful tools for multiplication and genetic transformation of plant material. Therefore, the development of biotechnological methodologies such as the *in vitro* regeneration protocols for

producing transgenic single-cell C_4 plants will provide us with powerful experimental tools to further enhance our understanding of this fascinating species.

1.2 Photosynthesis

Almost all life on earth relies on the products of photosynthesis for chemical energy. Chloroplasts in algae and plant cells harvest light energy through a series of reactions that convert it to chemical energy. This chemical energy is then used to assimilate atmospheric carbon dioxide into organic molecules such as glucose (Cheng and Fleming, 2008, Tipple and Pagani, 2007). Photosynthesis can be summarized by the following overall reaction:

$$6H_2O + 6CO_2 + \text{energy} \rightarrow 6O_2 + C_6H_{12}O_6$$

There are three main types of photosynthesis: C_3, C_4, and Crassulacean acid metabolism (CAM). C_3 is the most common, albeit least specialized, form of photosynthesis in terrestrial plants (Tipple and Pagani, 2007). C_4 and CAM are "add-ons" adaptations to the C_3 cycle in some angiosperms to better survive in hot, dry, arid environments.

All modes of photosynthesis rely on the efficiency of the primary CO_2 assimilation enzyme, Ribulose 1,5 bisphosphate carboxylase/oxygenase (Rubisco) to fix atmospheric carbon into usable chemical energy storage molecules (Patel and Berry, 2007). C_3 photosynthesis occurs within the mesophyll cells of the majority of plant species. It uses the Calvin-Benson cycle to convert atmospheric CO_2 into a 3-carbon molecule, glyceraldehydes-3-phosphate (G3P) (Tipple and Pagani, 2007). The activity of Rubisco is one of the major limiting factors in the efficiency of the Calvin-Benson cycle due to the enzyme's dual function as a carboxylase and oxygenase (Perry et al., 2007).

Despite Rubisco's high affinity for CO_2, in high-oxygen environments the plant will undergo photorespiration, a process that produces CO_2 and inhibits ribulose 1,5 bisphosphate cycling (Tipple and Pagani, 2007). This is a serious disadvantage that can drastically reduce plant growth and productivity, especially in arid environments when water and nutrients become limited. Rubisco is a slow-working enzyme, and is required in very high concentrations to be efficient. It is estimated that approximately half of the soluble protein in a leaf consists of Rubisco and it may still be limiting (Perry et al., 2007). Therefore, plants have continuously evolved ways to overcome the inefficiencies of Rubisco. To date, studies aimed at artificially improving crop yield involved modifying the specificity of Rubisco, creating CO_2 pumps to increase the amount of available carbon, and modifying the efficiency of the pathway (Perry et al., 2007).

CAM plants increase the efficiency of C_3 photosynthesis by assimilating atmospheric CO_2 at night when less water will be lost through transpiration (Cushman et al., 2008). This physiological process is highly efficient in desert or arid environments where unnecessary water loss can be detrimental to the plant. In CAM metabolism, carbon is fixed at night by phosphoenol pyruvate carboxylase (PEPC) into four-carbon acid and stored in the vacuole. During the day, the stored acid is then transported to the chloroplast to supply the CO_2 for the Calvin-Benson cycle. In CAM plants, key photosynthetic enzymes such as Rubisco and PEPC are regulated by circadian rhythms such that their peak activities occur at midday and midnight, respectively (Cushman et al., 2008). PEPC is mainly functional during the night when stomata are open and down regulated during the day when stomata are closed and malate (a negative regulator of PEPC) enters the cytoplasm.

In land plants, C_4 photosynthesis had previously been characterized by the presence of Kranz anatomy, or the dual-cell paradigm (Hatch, 1967). The function of the C_4 pathway involves the spatial compartmentalization of photosynthetic enzymes into two distinct cell types, the mesophyll and bundle sheath cells, to increase carbon fixation efficiency (Fig. 1.1A). This anatomical innovation spatially separates oxygen production from carbon dioxide assimilation to lower the O_2 concentration in the vicinity of Rubisco (Hibberd and Covshoff, 2010). Atmospheric carbon fixation via PEPC occurs in the mesophyll cells, and the Calvin-Benson cycle takes place in the bundle sheath cells where CO_2 is concentrated. In addition, the low oxygen environment created by the thick cell wall barrier of the bundle sheath cell limits Rubisco's oxygenase activity, thus increasing photosynthetic activity and minimizing photorespiration (Edwards et al., 2004). In the relative absence of oxygen, the carboxylase activity of Rubisco fixes CO_2 into a usable carbon source without wasting valuable energy catalyzing the breakdown of carbon intermediates via oxygenation (Edwards et al., 2004).

Recently, three plant species from the family Chenopodiaceae have been shown to have unique anatomical features allowing C_4 photosynthesis to take place in distinct cytoplasmic regions within a single chlorenchyma cell. The three species, *Bienertia sinuspersici*, *Bienertia cycloptera*, and *Suaeda aralocaspcia*, are C_4 plants, however they lack Kranz anatomy (Akhani et al., 2005, Freitag and Stichler, 2000, 2002, Voznesenskaya et al., 2001, 2002). Chlorenchyma cells in the leaves of these plants have evolved means of intracellular compartmentalization of organelles and enzymes to achieve functional C_4 photosynthesis within a single cell (Edwards et al., 2004).

Different single-cell C_4 anatomies are present in the *Bienertia* and the *Suaeda* systems (Fig. 1.1B and C).

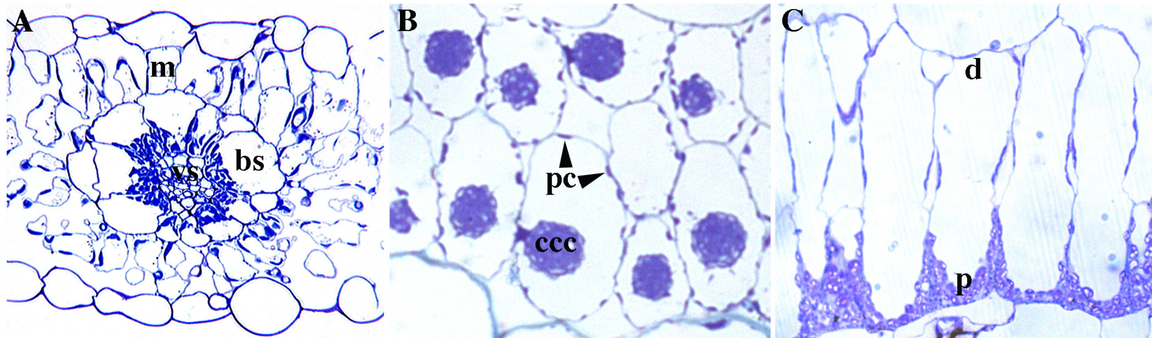

Figure 1.1 The three different C_4 photosynthetic pathways found in leaves of angiosperms.

(A) Most C_4 species (*Cleome gynandra*) have Kranz anatomy consisting of two distinct cell types, mesophyll cells (m) and bundle sheath cells (bs), that form a ring around vascular tissue (vs). Three members of the family Chenopodiaceae have the equivalents of the Kranz-type C_4 photosynthetic anatomy in a single cell. (B) In the *Bienertia* system, the C_4 cycles are compartmentalized in the peripheral cytoplasmic (pc) and central cytoplasmic compartment (ccc) of the cell. (C) In *Suaeda aralocaspica*, the C_4 cycles occur in different cytoplasmic regions (d = distal versus p = proximal) of the cell.

In the *Bienertia* system, photosynthetic enzymes and organelles are separated into a peripheral cytoplasmic compartment (pcc), the equivalent of the mesophyll cells in the dual-cell Kranz anatomy, and a central cytoplasmic compartment (ccc), the equivalent of the bundle sheath cell (Voznesenskaya et al., 2002; Fig. 1.1B). *Suaeda aralocaspica* possesses a completely different mode of single-cell C_4 photosynthesis; leaves of this species consist of elongated palisade chlorenchyma cells which partition photosynthetic enzymes and organelles at opposite ends of the cell (Voznesenskaya et al., 2001; Fig. 1.1C). This cellular arrangement is analogous to having dual cell Kranz anatomy without

intervening cell walls. Despite these differences, the same general biochemical features are present in *B. sinuspersici* and *S. aralocaspica* as those exhibited by other Kranz-type C_4 species (Edwards et al., 2004).

Since C_4 plants have higher water and nitrogen efficiency and increased yield compared to C_3 plants, it has been suggested that transforming C_3 crop plants with C_4 genes could increase carbon fixation efficiency and their yield (Hibberd and Covshoff, 2010). The discovery of single-cell C_4 photosynthetic systems have led to a re-examination of the anatomical requirements for C_4 photosynthesis being induced in C_3 crop plants. It may be much simpler to modify existing biochemical pathways of photosynthetic cells in C_3 crop plants to that of single-cell C_4 photosynthesis rather than changing the entire operation of C_3 mesophyll and bundle sheath cells to accommodate the dual cell paradigm. Such modifications to C_3 crop plants could revolutionize agriculture through increased yield.

1.3 Plant regeneration

In 1902, the phenomenon known as "totipotency" that proposed individual nucleated plant cells have the capacity to regenerate complete plants was introduced (Haberlandt, 1902). Since then, regeneration of plants has enabled researchers to transform and mass propagate whole plants *in vitro*. *In vitro* techniques exploit the totipotency of culture cells, tissues, and organs from explants or callus tissue in order to regenerate plants. Plant cells and tissues can grow on media containing inorganic salts, a carbon source, vitamins, and hormones or growth regulators (Murashige, 1974). One of the key factors to developing tissue culture protocols is the manipulation of hormones in

the growth media. Plant growth hormones are grouped into six major categories: auxins, cytokinins, gibberellins, brassinosteroids, abscisic acid, and ethylene, with auxins and cytokinins being the most important to tissue culture. Auxins and cytokinins differ from the other hormones in that they are required for plant viability. Thus, no mutants lacking auxin or cytokinin have been identified to date, implying that these hormones are essential.

Skoog and Miller reported the interactions of plant hormones in organ formation as early as 1956. The ratio of auxin to cytokinin has been shown to be essential in organ development because they interact to control cell division. Experiments performed by Skoog and Miller demonstrated that roots and shoots can be induced from callus cultures of tobacco by manipulating the ratio of auxin and cytokinin in the growth medium. A high auxin to cytokinin ratio promotes root development while a high cytokinin to auxin ratio promotes shoot development (Skoog and Miller, 1957).

In vitro regeneration has long been employed commercially for the propagation of plant material for agricultural, horticultural, pharmaceutical, or research purposes and for generating disease-free stock. There are several paths for plant regeneration including vegetative propagation via cuttings and organogenesis directly from plant explants or indirectly from undifferentiated callus tissue.

1.3.1 Vegetative asexual propagation

Vegetative asexual propagation provides a means for high-frequency replication of plant material through the use of cuttings. The nature of vegetative propagation relies on the fact that a branch of a plant may be excised and used as the starting material for

creating a genetically identical daughter plant. Adventitious root induction of cuttings has been established as a routine method of propagating many plants for their horticultural, agricultural, or pharmaceutical properties (Thorpe, 2007). It is efficient because time is not required to regenerate the shoot and stem *in vitro*. Growing new plants from seeds requires time for the seed to break dormancy, germinate, establish itself, and there is a chance that the seedling will be non-viable. Propagation from seeds may also be unfavorable due to the expression of undesired phenotypes. Plants produced through vegetative asexual propagation are genetically identical to the parent and therefore contain the same desired phenotypes (Kesari and Krishnamacheri, 2007). Vegetative asexual propagation is particularly advantageous for rare or important plants where a specific quality is desired in daughter plants, which is of particular value in the horticulture industry where plants are selected based on aesthetic traits like flower size or colour (Kesari and Krishnamacheri, 2007). For example, vegetative cuttings are used to reproduce many grape vines to maintain a single cultivar with a particular appearance, and flavour within a vineyard (This et al., 2006). Thus there is a sizeable increase in propagation efficiency by rooting cuttings compared to growing plants from seed.

In order to create a large stock supply of plants, cuttings of existing plants may be cultured in media containing auxins to induce adventitious root formation. Auxins were the first plant growth hormones discovered, and are generally known for their roles in plant growth and development including apical dominance, ethylene biosynthesis, cell elongation and differentiation, cell signaling, embryonic development, tropic responses, and senescence (Teale et al. 2006). The role of auxins in organ formation of roots, especially root induction and adventitious root formation, has made them invaluable in

the field of plant tissue culture (Kesari and Krishnamacheri, 2007). Rooting is primarily regulated by a high auxin to cytokinin ratio, therefore root formation can be initiated by adding an auxin to the growth medium (Skoog and Miller, 1957). Natural and synthetic auxins commonly used in tissue culture include 2,4 dichlorophenoxyacetic acid (2,4-D), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), and α-naphthaline acetic acid (NAA).

1.3.2 Direct Organogenesis

Direct organogenesis refers to the manipulation of differentiated plant tissue through subcellular changes to produce new shoots or roots (Hicks, 1980). Micropropagation, the process of producing large quantities of plants through tissue culture, enables whole-plant regeneration *in vitro*. Through direct organogenesis, several thousand clones of a single parent plant can be generated in a short time frame, and the maintenance of actively dividing shoots through subculturing can provide a reliable source of new plantlets (Akin-Idowu et al., 2009). Direct organogenesis typically involves the culturing of explants on a medium supplemented with nutrients and hormones amenable to shoot development. One type of direct organogenesis is axillary bud culture, which involves culturing excised apical and axillary buds on replication media to multiply existing shoots (Akin-Idowu et al., 2009). Similar to vegetative propagation, direct organogenesis provides a method to maintain genetic uniformity in important agricultural or horticultural cultivars (Sharma et al., 2007). For examples, potatoes are generally propagated through axillary bud culture because it is faster to get many minitubers from micropropagation techniques than it is to grow them individually

from seed, and to maintain important agronomic traits (Sharma et al., 2007). Similarly, commercial roses are micropropagated from axillary buds or nodal segments to produce desirable phenotypes, healthy, disease-free plants with shorter, more compact shoots, faster growth, and earlier flowering (Pati et al., 2006).

Apical and axillary buds may be cultured on media containing cytokinins in order to induce multiplication of shoots. Following high frequency multiplication, *in vitro*-derived shoots must be elongated in order to create an auxin gradient for further developmental regulation, and to protect the plant from bacterial stress and water damage once it has been transplanted to soil. Elongation is often induced by lowering the concentration of cytokinin in the media to create a smaller cytokinin:auxin ratio, or by adding gibberellic acid (GA_3) to the growth medium. Gibberellins are phytohormones with many impacts on plant growth including the regulation of flowering and the elongation of shoots (Marth et al., 1956).

Since each plant species has a unique physiology with its own preferred growth requirements, signaling responses, endogenous growth regulators, and metabolic capabilities, approximate nutrient media and hormones must be empirically determined. Protocols must be optimized to the specific growth requirements of a given plant to determine the most efficient way to propagate that plant *in vitro* (Akin-Idowu et al., 2009).

This *in vitro* technique provides a relatively efficient way of mass propagating plants without the need of employing a callus intermediate stage (Akin-Idowu et al., 2009). Since callus cells divide rapidly, they are subject to somaclonal variation through chromosomal rearrangement or mitotic mistakes. Therefore, in order to prevent genetic

variability often associated with *in vitro* procedures, it has been recommended that the period of time the cell spends in a callus stage be minimized or eliminated entirely (Hicks, 1980).

1.3.3 Indirect Organogenesis

Callus tissue is an undifferentiated, rapidly growing mass of cells that forms when plant tissue is wounded. Organogenesis may be induced indirectly from stem or leaf explants through the production of callus tissue by using a cytokinin, such as 6-benzylaminopurine (BAP), thidiazuron (TDZ), or kinetin, to differentiate callus cells into new shoots (Thorpe, 2007). Callus cells may respond to the cytokinin by inducing new shoots, resulting in high-frequency regeneration (Akin-Idowu et al., 2009).

The initial step of indirect organogenesis is the formation of callus tissue from the plant of interest. The effectiveness of explants to produce calli depends on the optimization of media compositions and plant growth regulators. Callus tissue may be induced by culturing explants on media supplemented with a moderate concentration of auxins and cytokinins to stimulate growth (Akin-Idowu et al., 2009). The callus may then be cultured on a shoot induction medium containing cytokinin to begin the regeneration protocol. Once shoots have been induced, they may be propagated in the same manner as excised apical or axillary buds in direct organogenesis.

Whole-plant regeneration has always been a key step in generating transgenic plants. A preferred method of generating a transgenic plant is by inserting a DNA plasmid into a single, totipotent cell such that the cell may integrate the gene of interest and be competent for indirect organogenesis (Eady, 1995). Natural variation occurring in

the callus tissue resulting from the explant source and culture conditions influence their regeneration potential. Thus, not all callus cells that exhibit totipotency will be able to undergo regeneration (Kondamudi et al., 2009). In onions, regeneration of callus was more efficient when the callus was derived from embryos than from vegetative tissues such as bulbs or stems, and therefore researchers have relied on the high totipotency of embryo-derived callus for transformation studies (Eady, 1995). For successful transformation to occur, the cell must be able to take up the desired DNA and express the newly inserted gene when grown using a whole-plant regeneration protocol. To determine whether or not the desired DNA has been taken up, transformed calli may be identified using a selectable marker, such as an antibiotic or herbicide, to isolate cells that contain the introduced gene (Eady, 1995).

1.4 Objectives and Hypothesis

The continuous supply of plants and plant tissue that will be required by other lab members incites the need for an *in vitro* regeneration protocol for *B. sinuspersici*. Although plant tissue culture is a well-established area of research, there is a constant need to optimize established methods due to demand for more efficient *in vitro* regeneration methods and the discovery of new plant species that require optimized protocols. Despite the potential agricultural importance of the single-cell C_4 photosynthesis, *in vitro* regeneration protocols have not been developed for *B. sinuspersici* to date. Thus, the establishment of these efficient *in vitro* protocols would enable researchers to regenerate plants *in vitro* and create transformation systems that

will facilitate our understanding of the cellular and molecular mechanisms controlling single-cell C_4 photosynthesis.

The overall goal of this master's thesis was to develop an efficient and reliable *in vitro* multiplication and regeneration system for *B. sinuspersici*. Propagation methods involving vegetative asexual propagation of cuttings, axillary bud culture, and indirect organogenesis have been employed to achieve this goal. It was hypothesized that explants of *B. sinuspersici* cultured on growth medium containing appropriate concentrations of phytohormones would result in higher regeneration efficiency than explants on hormone-free media.

Chapter 2

Materials and Methods

2.1 Materials

All chemicals were purchased from BioShop (Ontario, Canada) unless otherwise indicated.

2.1.1 Plant material

Seeds of *Bienertia sinuspersici* were germinated on moist filter paper in Petri dishes at room temperature. Seedlings were transplanted to 11 cm pots containing Sunshine Mix #4 potting soil (Jack Van Klavereen, Ontario, Canada) and grown for one week at room temperature under 60 μmol m^{-2} s^{-1}. Plants were then grown in a growth chamber (model GCW-15H; Environmental Growth Chambers, Ohio, USA) under 350 μmol m^{-2} s^{-1} with a 14 h/10 h light/dark photoperiod and a 25/18°C day/night temperature regime. Plants were watered every 3 days and fertilized with Miracle-Gro fertilizer (24-8-16; Scott's Miracle-Gro Company, Ontario, Canada) once a week. Plant samples used for propagation in this project were taken from these original plants.

2.2 Methods

2.2.1 Media preparation

All *in vitro* manipulations were conducted in a laminar flow hood under sterile conditions. All plant materials were cultured on MS medium. Full strength MS media consisted of 4.32 g L^{-1} MS basal medium with Gamborg's vitamins (Sigma-Aldrich,

Ontario, Canada), 1.96 g L^{-1} MES, 30 g L^{-1} sucrose, and a solidifying agent of either 7 g L^{-1} agar or 3.5 g L^{-1} gelzan (PlantMedia, Ohio, USA). The media was adjusted to pH 5.8 before adding solidifying agent and then sterilized by autoclaving at 121ºC, 1.1 kg cm^{-2} for 30 minutes. After autoclaving, the media was allowed to cool to approximately 60ºC before heat sensitive components such as hormones were added. All plant hormones were purchased from Sigma-Aldrich and prepared as 1 mg mL^{-1} working stock solutions [auxins in hydrochloric acid; cytokinins and gibberellic acid in ethanol], filter sterilized through a 0.2 µm filter, and stored at -20˚C. Approximately 30 mL of media were poured into each sterile Petri dish (100 x 15 mm) or 60 mL per Magenta box (GA7; Magenta Corp., Illinois, USA) and stored at room temperature for a least 3 days prior to use.

The composition of the growth medium varied based on the type of explants and experimental objective. Callus induction, shoot induction, multiplication, and elongation experiments were all conducted on full strength MS medium supplemented with 30 g L^{-1} sucrose. The amount of MS and sucrose in the medium was halved for the root induction experiments. Cuttings were cultured on 1/2 MS medium (see section 2.2.3). Agar was used as a solidifying agent for shoot replication, elongation, and rooting, while gelzan was used for callus induction and shoot induction from callus.

2.2.2 Explant sterilization

Plant material was surface sterilized for 10 min in 0.5% (v/v) sodium hypochloride solution followed by two 3 min rinses in sterile distilled water.

2.2.3 Induction of roots on stem cuttings

For root induction experiments from *B. sinuspersici* cuttings, cuttings (5 – 8 cm) were obtained from 6- to 8-month old mature plants. Leaves were removed from the bottom 2 cm of stem before cuttings were surface sterilized as described in Section 2.2.2. Four cuttings were placed in each Magenta box containing various concentrations of MS medium (quarter-, half-, three quarter-, or full-strength). Root induction of cuttings was also performed on half-strength MS medium supplemented with auxins (IAA, IBA, NAA, and 2,4-D) at 0.5, 1.0, 2.5, and 5.0 mg L^{-1}. Cuttings were maintained in a growth chamber (Percival Scientific, Iowa, USA) with a 14 h/10 h light/dark photoperiod at 22°C under 25 µmol m^{-2} s^{-1}. After 3 weeks, rooted cuttings were carefully removed from the agar, gently washed in water, photographed, and the number and length of roots was recorded for each plant before transplanting to 10 cm pot containing Sunshine mix #4 potting soil. Transplanted cuttings were maintained in a humidifying dome and gradually acclimatized to greenhouse conditions. Plant survival rate was measured after 3 weeks.

Cuttings of mature plants were also cultured on medium containing GA_3 (0.1, 1.0, 3.0, and 5.0 mg L^{-1}) to examine shoot elongation. Stem cuttings were measured to specific lengths (0, 5, 10, 20, 30, 40, and 50 mm) with the condition of 0 mm stem length plants consisting of only the apical shoot tip. After 3 weeks, the shoot length and the number of rooted plants were measured and recorded. Three replicates, each containing five cuttings, were carried out for each treatment.

2.2.4 Induction of callus on explants

Primary callus induction was initiated by culturing stem or leaf explants on callus inducing medium (CIM) consisting of MS salts with Gamborg's vitamins that contained various combinations of auxins (2,4-D, NAA; 0.5, 1.0, and 2.5 mg L^{-1}) and cytokinins (BAP, kinetin; 0.5, 1.0, and 2.5 mg L^{-1}). Explants were taken from mature plants, sterilized, aseptically cut into 0.5 cm sections, and placed on CIM. Explants were gently pressed into the surface of the medium allowing approximately half of each explant to be in contact with the media. Sealed plates were wrapped in aluminum foil to create dark conditions and were maintained in a growth chamber at 22˚C. Explants were examined at 2, 4, and 6 weeks intervals. At least three replicate dishes were used for each of the treatments, each containing 20 explants. The number of responsive explants was recorded and imaged using a Zeiss stereomicroscope (Stemi SV11; Carl Zeiss Canada Ltd., Ontario, Canada). Images were captured using a cooled CCD camera (Retiga 1350 Exi Fast, Qimaging, British Columbia, Canada) and the OpenLab (OpenLab, Ontario, Canada) imaging software. The health of the callus was also assessed. Calli were continuously subcultured on CIM every 6 to 8 weeks and maintained in the dark.

2.2.5 Direct organogenesis

Apical and axillary buds were obtained from 6- to 8-month old plants. Outer leaves were removed such that each bud only contained one or two of the innermost leaves. Excised buds were surface sterilized, plated on Petri dishes containing full strength MS medium supplemented with various concentrations of BAP (0.5, 1, 2, 4, 6, 8, 10, 15, or 20 mg L^{-1}) and maintained in a growth chamber with a 14 h/10 h light/dark

photoperiod at 22°C under 25 μmol m^{-2} s^{-1}. The number of responsive shoots and number of new shoots formed were counted after 3 weeks. The *in vitro*-derived shoots were then transferred to elongation media containing either gibberellic acid alone (GA_3; 0.1, 1.0, 3.0, and 5.0 mg L^{-1}) or to media containing a low concentration of cytokinin (BAP; 0.001, 0.002, and 0.005 mg L^{-1}) in combination with an auxin (NAA or IAA; 0.0005, 0.001, and 0.0025 mg L^{-1}), or to media containing gibberellic acid (0.00037, 0.0037, and 0.011 mg L^{-1}) in conjunction with thidiazuron (TDZ), a synthetic cytokinin (0.000025, 0.00005, and 0.000075 mg L^{-1}). The height of shoots was measured at the time of plating and again after 5 weeks to determine the amount of elongation induced.

Elongated shoots were then transferred to Magenta boxes containing MS medium supplemented with various concentrations (0.5, 1.0, 2.5, and 5 mg L^{-1}) of IBA to induce rooting or the cut end was dipped in 0.1% (w/v) rooting powder (Sure-Grow, Ontario, Canada) and transferred to an Oasis Rooting Medium (Smithers-Oasis, Ohio, USA). Alternatively, elongated shoots (scions) were grafted to *in vitro*-derived roots from cuttings (stocks) by making a "v" shaped cut in both the stem of the scion and stock such that they fit snugly together. The micrografted stock and scion plants were wrapped tightly with a thin strip of aluminum foil to hold them in place, and maintained in a humidifying dome at room temperature under 60 μmol m^{-2} s^{-1}.

In vitro-derived rooted shoots were transplanted to soil and acclimatized in the greenhouse under a humidifying dome prior to being transferred to a growth chamber under 350 μmol m^{-2} s^{-1} with a 14 h/10 h light/dark photoperiod and a 25/18°C day/night temperature regime.

2.2.6 *Indirect organogenesis*

Calli used for the indirect organogenesis experiments were derived and maintained on CIM medium containing 2.5 mg L^{-1} 2,4-D and 0.5 mg L^{-1} kinetin. Three cytokinins were used for shoot induction: BAP and kinetin were applied at 0.5, 1.0, 5.0, and 10 mg L^{-1} whereas TDZ was used at 0.001, 0.005, 0.01, 0.1, 0.25, 0.5, 0.75, 1.0, and 2.0 mg L^{-1}. Calli were cultured and maintained in a growth chamber with a 14 h/10 h light/dark photoperiod at 22˚C under 25 μmol m^{-2} s^{-1}. After 6 weeks, calli exhibiting the formation of pre-shoots (green, smooth clusters of cells that are beginning to form shoot primordia) were observed. Responsive calli were subcultured every 2 or 3 weeks onto fresh shoot induction media until true shoots were completely developed. Shoots were then multiplied by sub-culturing on media containing 2 mg L^{-1} BAP. Elongation and root induction of shoots derived from indirect organogenesis were performed concurrently with those derived from the direct organogenesis experiments (see section 2.2.5).

2.2.7 *Light Microscopy*

Leaves, roots and stems were prepared for general microscopy. Samples were fixed in 2% (v/v) paraformaldehyde and 2% (v/v) glutaraldehyde (Electron Microscopy Sciences [EMS], Pennsylvania, USA) in 50 mM PIPES buffer, pH 7.2 overnight at 4˚C. The samples were dehydrated with a graded ethanol series (30%, 50%, 60%, 70%, 80%, 95%, 95%, 100%, 100%) for 30 min each at room temperature, gradually infiltrated with increasing concentrations of London Resin White (LR white; EMS, Pennsylvania, USA) acrylic resin, and embedded in pure LR white overnight at 60˚C. The polymerized resin samples were cut, mounted onto mounting cylinders (Ted Pella Inc., California, USA),

and trimmed. Sections (1 μm) were prepared on a Reichert Ultracut E ultramicrotome (Reichert-Jung, Heidelberg, Germany), dried onto glass slides, and stained with 0.1% (w/v) Toluidine blue (TBO) (Sigma-Aldrich, Ontario, Canada). For freehand sections, fresh leaves were obtained from shoots in Magenta boxes or from plants in a growth chamber. Sections (~200 μm) were made using a double edge stainless steel PERSONNA razor blade (EMS, Pennsylvania, USA) and immediately fixed in 2% (v/v) paraformaldehyde and 2% (v/v) glutaraldehyde (EMS, Pennsylvania, USA) in 50 mM PIPES buffer, pH 7.2. Plastic or freehand sections were observed using a Zeiss Axiophot (Carl Zeiss Canada Ltd., Ontario, Canada) light microscope. Images were captured using a cooled CCD camera (Retiga 1350 Exi Fast, Qimaging, British Columbia, Canada) and OpenLab (OpenLab, Ontario, Canada) imaging software. Image processing was performed using Adobe Photoshop CS (Adobe, California, USA).

2.2.8 Extraction of total proteins from plant tissues

Leaves or stems were collected, frozen in liquid nitrogen, and stored at -80˚C prior to use. Total proteins were extracted from plant tissues by homogenizing in liquid nitrogen to a fine powder with a mortar and pestle. The powderized samples were further ground in extraction buffer (100 mM Tris-HCl, pH 7.5, 10 mM EDTA, 1% [v/v] Triton X-100, 1 mM DTT, 10 μL mL^{-1} protease inhibitor cocktail [Sigma-Aldrich,Ontario, Canada], 1 mM PMSF) with a pinch of sea-washed sand (Fisher Scientific, Ontario, Canada). The homogenates were centrifuged at 14,000 rpm for 5 min at 4˚C and the supernatants were carefully transferred to fresh tubes on ice and used for quantification of protein concentrations. The concentrations of protein were determined according to the

procedure described by Bradford (1976). Briefly, 2 µl of protein extract was mixed with 498 µl of Bradford reagent (BioRad, Ontario, Canada), incubated for 5 min. and the absorbance at 595 nm was measured against a blank prepared from 2 µl of the extraction buffer and 498 µl of Bradford reagent. Bovine serum albumin (Sigma-Aldrich, Ontario, Canada) was used as a standard in a range between 1 to 10 µg.

2.2.9 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Discontinuous gels (for the stacking gel: 4% [w/v] acrylamide/bisacrylamide 37.5:1, 125 mM Tris-HCl, pH 6.8; for the separating gel: 10% [w/v] acrylamide/bisacrylamide 37.5:1, 375 mM Tris-HCl pH 8.8, 0.1% [w/v] SDS, 0.05% [w/v] ammonium persulphate, and TEMED) were used to separate proteins under denaturing conditions according to Laemmli (1970). Protein samples (15 µg) were mixed with 5x SDS-PAGE sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% [v/v] glycerol, 2% [w/v] SDS, 0.05 % [w/v] bromophenol blue, 5% [v/v] β-mercaptoethanol), heated at 95 ˚C for 5 min, and spun for 5 sec. The boiled samples were loaded into submerged lanes containing 1x SDS-PAGE electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1% [w/v] SDS, pH 8.3) and electrophoresed at a constant voltage of 120 V for 45 min or until the bromophenol blue dye front reached the bottom of the gels. The Bio-Rad Mini PROTEAN Tetra Cell (BioRad, Ontario, Canada) apparatus was used for all electrophoresis. After electrophoresis, the separated proteins were either stained with Coomassie Blue R-250 (0.2% [w/v] Coomassie Blue, 7.5% [v/v] acetic acid, and 50% [v/v] methanol) to visualize protein bands or used for Western blot analysis.

2.2.10 Western blot analysis

For western blot analysis, separated proteins were transferred to polyvinylidene difluoride (PVDF) (Roche, Ontario, Canada) or nitrocellulose (BioRad, Ontario, Canada) membrane using the Bio-Rad semi-dry transblotter (Bio-Rad, Ontario, Canada). The membrane and filter paper were cut according to the gel dimensions. PVDF (pre-wetted in methanol) or nitrocellulose membrane, filter paper, and SDS-PAGE gel were incubated in transfer buffer (48 mM Tris, 39 mM glycine, 0.001% [v/v] SDS, 20% [v/v] methanol) for 20 min. The gel sandwich assembly consisting of filter paper, membrane, gel, and filter paper was assembled according to the manufacturer procedure. Protein transfer was then performed at 20 V for 40 min. After the transfer, the membrane was briefly stained with Ponceau S stain (0.1% [w/v)] Ponceau S in 5% [v/v] acetic acid) to determine whether proteins had been effectively transferred, then rinsed thoroughly with distilled water, and incubated in blocking buffer (5% [w/v] skim milk in TBS-T buffer: 25 mM Tris-HCl pH 7.4, 137 mM NaCl, 27 mM KCl, 0.1% [v/v] Tween 20) for 1 h at room temperature. The blocked membrane was incubated with the appropriate primary antibody in blocking buffer overnight at 4˚C with gentle shaking. The primary antibodies used were: anti-RbcL (antibody against the large subunit of Rubisco Form I and Form II raised in rabbit; Agrisera, Vannas, Sweden, diluted 1:10 000), anti-PEPC (antibody against maize PEPC raised in rabbit; Chemicon, California, USA, diluted 1:20 000), anti-NAD-ME (antibody against the α-subunit NAD-ME of *Amaranthus,* courtesy of James Berry, SUNY, New York, USA, diluted 1:4 000), and anti-PPDK (antibody against maize PPDK, courtesy of Chris Chastain, MNSU, Minnesota, USA, diluted 1:10 000). After an

overnight incubation, the membrane was rinsed 3 times in TBS-T buffer for 15 min each and incubated with the secondary antibody goat anti-rabbit IgG horseradish peroxidase (HRP) conjugated (Sigma-Aldrich, Ontario, Canada, diluted 1:10 000) for 2 h at room temperature with gentle shaking, and then washed again 3 times with TBS-T for 15 min each. The membrane was incubated in ECL solution (1.25 mM Luminol, 2 mM *p*-coumaric acid, 5.3 mM H_2O_2 in 100 mM Tris-HCl, pH 8.8) in the dark for 5 min. Luminescence was detected by exposing the membrane to Amersham hypersensitive film (GE Health Care, Ontario, Canada) and developed using a CP1000 Agfa photodeveloper. (AGFA, Ontario, Canada). The film was scanned and processed using Adobe Photoshop CS (Adobe, California, USA).

2.2.11 Statistical Analysis

Statistical analysis of data was performed with Statistical Package for the Social Sciences (SPSS) version Predictive Analysis SoftWare (PASW) (SPSS, Illinois, USA) using one or two-way ANOVAs with post-hoc Tukey tests. ANOVA tests analyze the variance between two or more groups to see if they have the same mean (Dytham, 2003). The test generates an F statistic and a significance value. The F value compares the ratio of variance within the groups to the variation between the groups. If the variance between the groups is larger than the variance within the group, their means are significantly different. If the significance value is less than 0.05, the means between the groups are significantly different at the 95% level (Dytham, 2003). Univariate (one-way) ANOVAs test data with one independent variable (concentration), multivariate (two-way) ANOVAs test data with two independent variables (multiple hormones used at multiple

concentrations). Post-hoc Tukey test were performed to determine which groups mean values differed significantly from those of other groups. ANOVA tests assume that the data is continuous and approximately normally distributed (Dytham, 2003). Normal distribution was analyzed using the Levene's test. Descriptive statistics were also generated using Microsoft Excel (Microsoft Corporation, Washington, USA).

2.2.12 Cytological analysis of the regenerated plants

Excised root tips (~1.5 cm long) from regenerated plantlets via indirect organogenesis were processed to determine their chromosome number. The root tips were pretreated with saturated paradichlorobenzene for 3 h at room temperature followed by overnight fixation in ethanol : acetic acid (3:1, v/v). Fixed root tips were hydrolyzed for 30 min in 1 M HCl at 60°C and stained with 2% aceto-orcein. The stained root tips were squashed in 45% (v/v) acetic acid onto a slide and examined under a microscope (Owen et al., 2006). Cells showing the metaphase stages were analyzed for chromosome number and photographed with a Nikon Coolpix 990 digital camera. Ten root tips were squashed for each *in vitro* derived shoots via indirect organogenesis and vegetative cuttings.

Chapter 3

Vegetative propagation of *Bienertia sinuspersici*

3.1 Introduction

Rooting of cuttings has been established as an efficient means of vegetatively propagating plant material. Vegetative or asexual propagation operates on the principle that any branch of a mature plant may be excised for adventitious root induction and subsequently grown into a new plant (Thorpe, 2007). Asexual reproduction provides a desirable method of plant propagation because it results in lower genetic variation between plants, therefore creating a homogenous population of plant material for research (Kesari and Krishnamacheri, 2007). Vegetative propagation by cuttings is advantageous because the rooted cuttings can serve as potential rootstocks for grafting experiments and for root protein extraction. Roots grown in agar are more suitable for root protein extraction because they are more easily removed from the substrate without damage and carry fewer contaminating organisms. Cuttings may also be used to determine the minimum stem length required for *in vitro*-derived plants to form roots. Propagation by cuttings is limited by the availability of mature plant material explant generation, as well as the inability to transform entire plants (Thorpe, 2007).

Rooting of cuttings is regulated by auxins, the first plant hormone discovered, known for their role in many aspects of plant development (Teale et al. 2006). The induction of adventitious roots of cuttings is influenced by the ratio of auxin to cytokinin present. In general, root induction can be initiated by adding an auxin to the growth medium (Skoog and Miller, 1957). Common auxins used for root induction *in vitro*

include 2,4 dichlorophenoxyacetic acid (2,4-D), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), and α-naphthaline acetic acid (NAA).

In this chapter, results were obtained using cuttings of *B. sinuspersici* that were subjected to various strengths of MS medium and different concentrations of auxins to determine their impact on root induction. Analysis of root anatomy was performed to examine the effect of various auxins on root development in *B. sinuspersici*.

3.2 Results

3.2.1 Effect of MS concentration on rooting of cuttings

A simple method for the vegetative propagation of *B. sinuspersici* plants via cuttings is described and represents the first reported method for regeneration of *B. sinuspersici* plants via cuttings. The development of a simple and effective plant regeneration protocol provides the means to produce abundant material for research purposes, by investigating the effects of MS basal medium strength, gelling agents, and auxins on the efficiency of adventitious root formation.

Due to the lower exogenous nutrient requirements of explants that are more resilient to *in vitro* conditions, full strength MS basal salts (4.32 mg L^{-1}) are not always required (Murashige and Skoog, 1957). After 3 weeks of culture, adventitious roots were observed on the cuttings in all treatments (Table 3.1). There was no significant difference in the number of roots formed among the different MS-containing media, however the presence of half- or three quarter-strength MS basal salts in the medium improved root length significantly. The highest number of roots were initiated in the treatment containing quarter-strength MS, while three-quarter-strength MS produced the

longest roots. Media containing half-strength MS appeared to produce a compromise between root length and number, and rooted cuttings from this treatment established themselves successfully in soil after transplantation.

Table 3.1: Effect of MS concentration on root induction of *B. sinuspersici* cuttings after 3 weeks in culture.

MS Strength	Number of roots / cutting	Root length (cm)	Survival rate (%)
0 MS	5.9 ± 2.5	3.2 ± 1.9	41.4 ± 20.0[a]
¼ MS	10.0 ± 2.2	3.6 ± 2.4	100.0 ± 0.0[b]
½ MS	9.4 ± 3.0	6.3 ± 3.8	100.0 ± 0.0[b]
¾ MS	6.0 ± 3.1	9.8 ± 2.8	71.4 ± 10.0[a]
1 MS	8.6 ± 2.4	4.9 ± 2.2	71.4 ± 10.0[a]

One-way ANOVA tests indicate that for both number of roots and root length, the difference in means (± SE) between the groups are much lower than those within the groups, suggesting that the treatments do not have significantly different means at the 95% confidence level, with test statistics of (F=1.59, sig. 0.20, n=3) and (F=0.72, sig. 0.58, n=3) respectively. Survival rate between treatment groups differs significantly at the 95% level (F=3.24, sig=0.01, n=3). Different letters in a column indicate a significant difference between treatments at $P \leq 0.05$ according to Tukey's test.

Rooted cuttings were removed from media, transplanted to soil, and acclimatized to greenhouse conditions. The survival rate of the transplanted cuttings was determined after three weeks. Survival rates of rooted cuttings differ significantly between the

control (0 MS) and the ¼ MS and ½ MS treatments, but not the ¾ MS or 1 MS treatments (Table 3.1).

3.2.2 Rooting of cuttings on various auxins

To further optimize the rooting of cuttings, half-strength MS media was supplemented with various auxins.

Table 3.2: Effect of different auxins on the induction of adventitious roots from cuttings after 3 weeks in culture.

Hormone	Concentration (mg L^{-1})	Number of Roots	Root Length (cm)	Survival Rate (%)
2,4-D	0	15.3 ± 4.8^{a}	23.8 ± 7.5^{a}	44.3 ± 0.1^{a}
	0.5	10.4 ± 3.2^{a}	1.8 ± 0.5^{b}	0.0 ± 0.0^{d}
	1.0	13.4 ± 3.7^{ab}	2.0 ± 0.7^{b}	50.0 ± 0.0^{c}
	2.5	5.6 ± 2.7^{ab}	1.4 ± 0.5^{b}	25.0 ± 0.06^{c}
	5.0	1.7 ± 1.7^{b}	0.5 ± 0.4^{b}	17.0 ± 0.04^{d}
IAA	0	8.3 ± 2.8^{a}	21.4 ± 8.8^{a}	100.0 ± 0.0^{a}
	0.5	21.0 ± 5.0^{a}	2.7 ± 0.5^{b}	50.0 ± 0.12^{ac}
	1.0	23.1 ± 4.1^{a}	3.4 ± 0.6^{b}	49.5 ± 0.04^{abcd}
	2.5	28.3 ± 8.2^{ac}	2.9 ± 0.6^{b}	83.0 ± 0.04^{bc}
	5.0	21.8 ± 7.6^{ac}	3.4 ± 0.6^{ab}	17.0 ± 0.04^{cd}
IBA	0	12.3 ± 1.9^{a}	25.3 ± 6.5^{a}	66.0 ± 0.0^{a}
	0.5	14.3 ± 1.9^{a}	27.3 ± 6.1^{a}	83.0 ± 0.04^{ab}
	1.0	9.1 ± 1.6^{a}	29.4 ± 7.0^{a}	100.0 ± 0.0^{a}
	2.5	10.7 ± 0.3^{a}	25.3 ± 6.7^{a}	100.0 ± 0.0^{a}
	5.0	14.8 ± 3.7^{a}	15.0 ± 5.6^{a}	100.0 ± 0.0^{a}
NAA	0	11.1 ± 3.3^{a}	22.3 ± 7.1^{a}	100.0 ± 0.0^{a}
	0.5	15.5 ± 4.4^{a}	6.7 ± 2.6^{ab}	100.0 ± 0.0^{a}
	1.0	19.4 ± 6.0^{a}	6.1 ± 3.0^{ab}	50.0 ± 0.0^{c}
	2.5	16.5 ± 8.4^{ac}	6.4 ± 4.7^{ab}	66.0 ± 0.0^{ab}
	5.0	44.2 ± 13.6^{c}	1.3 ± 0.4^{b}	77.3 ± 0.03^{ab}

Values represent means (± SE) from five independent experiments. Root length, root number, and survival rate each differ significantly at the 95% level ($P \leq 0.05$) for each treatment (F=4.40, sig=0.00, n=5), (F=4.09, sig=0.00, n=5), and (F=84.6, sig=0.00, n=5), respectively. Different letters represent statistically significant mean separations according to Tukey's test.

Table 3.2 shows that the addition of 1 mg L^{-1} IBA was most successful at inducing roots from cuttings. The addition of IBA to the growth medium produced several long roots with many lateral roots and root hairs (Fig. 3.2C). These roots appeared healthy and had a high success establishing the plant in soil. Cuttings with fewer, longer healthy roots were more predisposed to survival after transplantation compared to those with many short roots. For example, cuttings that were cultured on 5 mg L^{-1} NAA produced the highest number of roots, but roots were very short and the plants were were unable to successfully establish in soil, whereas cuttings treated with IBA produced fewer, longer roots and had higher survival rates after transplantation (Table 3.2). 2,4-D treatment produced a few short, feather-like aerial roots on cuttings just above the agar surface (Fig. 3.2E). The short, unhealthy roots coupled with the low rate of induction and survival makes 2,4-D a poor candidate for *in vitro* rooting study. Similarly, cuttings cultured on media supplemented with IAA formed clusters of many short and thick roots at the base (Fig. 3.2B). IAA-rooted cuttings had moderate success upon transplanting, due to the quantity of roots and the stability provided by the compact root structure. Furthermore, the cluster of roots made it difficult to remove the agar without causing physical damage. Cuttings cultured on media containing NAA developed many short and stubby root protrusions that were accompanied by a few feathery roots above the surface of the media similar to those observed in the 2,4-D treatment (Fig. 3.2D). These cuttings with poorly developed roots were unable to establish well in soil.

It was generally observed that cuttings with many short roots had lower survival rates after transplantation, compared to those with fewer and longer roots. Even though

cuttings cultured on 1 mg L^{-1} IBA produced fewer roots than some of the other treatments, the roots were the longest and had the highest rate of survival after transplanting. Roots induced by IBA appeared much healthier and more viable than

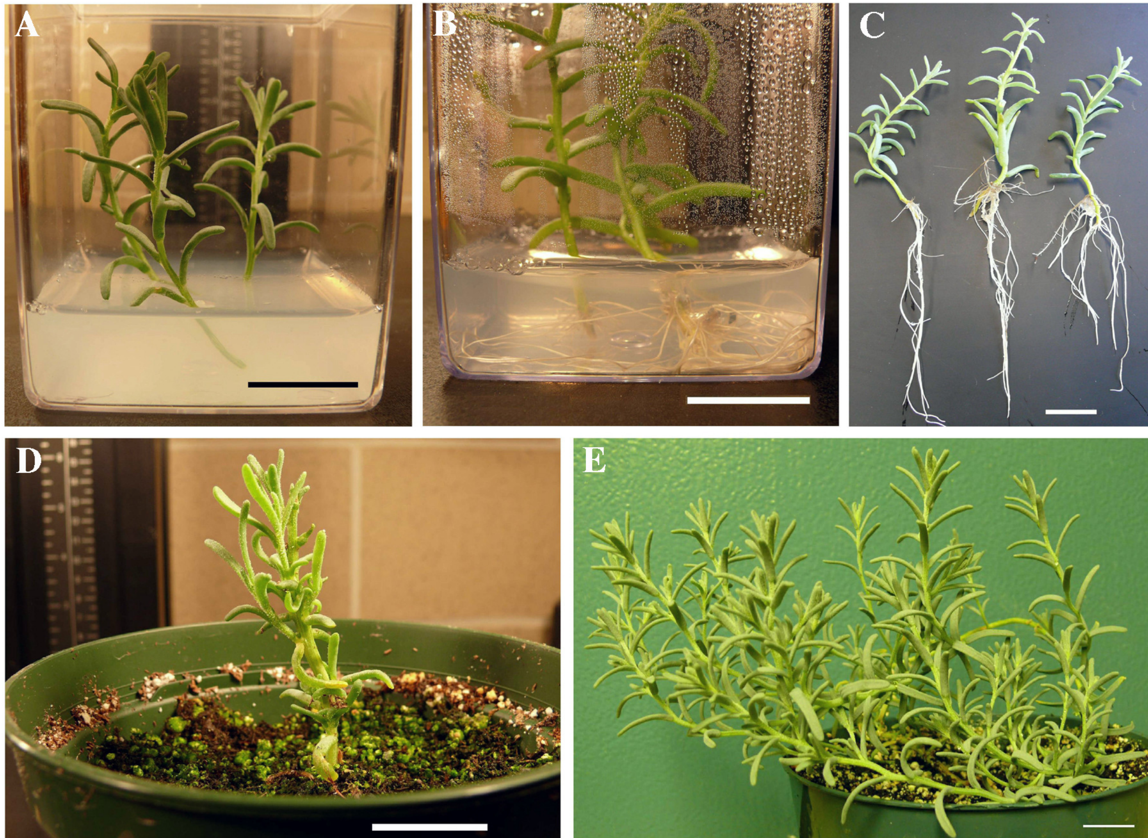

Figure 3.1 Induction of adventitious roots and plant regeneration in cuttings of *Bienertia sinuspersici*.

(A) 5 cm long cuttings of *B. sinuspersici* have been sterilized and planted in rooting medium. (B) Cuttings exhibit extensive root growth after three weeks in rooting medium. (C) Many long roots form on cuttings cultured on 1mg L^{-1} IBA. (D) After three weeks on soil, plants have fully acclimatized to greenhouse conditions. (E) *B. sinuspersici* plant derived from cuttings after three months in the greenhouse. Scale bars = 2 cm.

Figure 3.2 Effect of auxins on adventitious root formation and root anatomy in cuttings of *Bienertia sinuspersici.*

Analysis of root morphology and detailed longitudinal and tangential sections of roots further provide insight into root anatomy. Cuttings were rooted on (A and F) hormone-free medium and medium supplemented with (B and G) IAA, (C and H) IBA, (D and I) NAA, and (E and J) 2,4-D. Scale bars = 1 cm in A – E and 200 μm in F – J.

those produced in the hormone-free media. In addition, high survival rates after transplantation to soil further suggest IBA as the favorable candidate for *in vitro* root induction.

The anatomy of roots was analyzed to further examine the effect of auxins on the growth and development of roots in *B. sinuspersici*. The internal structure of a root generally consists of three well-defined overlapping regions commonly known as the zones of cell division, elongation, and maturation. Longitudinal sections of root tips on hormone-free media shows a thin root cap surrounding the root apical meristem that overlaps with the zone of elongation (Fig. 3.2F). Despite this, these roots appeared thin and slightly brown in colour, raising the question of whether supplementing the media with auxins would produce healthier roots. Histological examination of root tips showed that IAA-induced roots consist of many smaller cells that also organized into distinct regions (Fig. 3.2G). However, cells from the IAA-induced roots appeared to have a larger zone of cell division and were not elongating to produce long roots. Roots that were induced by IBA showed the best root anatomy as the various distinctive regions are clearly visible (Fig. 3.2H). Sections of root tips developed from the NAA treatment showed that each root tip consisted of many small cells organized into a condensed miniature root. Epidermal, cortical and root cap cells near the tip region appeared to be loosely packed and sloughed off prematurely (Fig. 3.2I). Longitudinal sections of 2,4-D induced root tips showed thin roots with irregularly shaped elongated parenchyma cells, a poorly developed root cap, and sloughing of epidermal cells (Fig. 3.2J).

3.2.3. Elongation of cuttings on GA_3

The effect of GA_3 on elongation and rooting of cuttings was examined to determine what effect stem length has on root formation in *B. sinuspersici* (Table 3.3). Data from Table 3.3 shows the effect of GA_3 on elongation response from different starting stem lengths in *B. sinuspersici*, and suggest that a minimum stem length of one to two centimeters will be sufficient to support root formation. While the objective of this experiment was to determine the required length for *in vitro*-derived shoots in order to induce roots, it may be noted that cuttings and *in vitro*-derived shoots may respond differently to these particular growth conditions.

Table 3.3: Effect of GA_3 on the elongation of shoots of *B. sinuspersici*.

	Shoot length (cm)						
GA concentration (mg L^{-1})	0	0.5	1.0	2.0	3.0	4.0	5.0
0	0.32 ± 0.08^{a}	1.11 ± 0.23^{c}	1.37 ± 0.07^{c}	2.31 ± 0.20^{d}	3.04 ± 0.11^{f}	4.14 ± 0.00^{h}	3.28 ± 0.00^{e}
0.1	0.91 ± 0.07^{b}	1.42 ± 0.11^{c}	2.49 ± 0.28^{d}	3.01 ± 0.22^{e}	3.87 ± 0.02^{g}	3.76 ± 0.02^{f}	4.20 ± 0.41^{g}
1.0	0.74 ± 0.17^{b}	1.79 ± 0.14^{c}	2.68 ± 0.33^{d}	2.99 ± 0.32^{e}	3.75 ± 0.64^{fg}	3.57 ± 0.02^{g}	4.90 ± 0.14^{h}
3.0	0.84 ± 0.11^{b}	1.71 ± 0.19^{c}	2.87 ± 0.19^{d}	3.28 ± 0.31^{e}	3.90 ± 0.42^{g}	3.85 ± 0.24^{g}	4.80 ± 0.00^{i}
5.0	0.81 ± 0.19^{b}	1.33 ± 0.31^{c}	2.58 ± 0.18^{d}	2.87 ± 0.40^{e}	4.32 ± 0.77^{g}	3.01 ± 0.17^{f}	4.96 ± 0.26^{hi}

Multivariate ANOVA values indicate that the mean (± SE) difference between the groups was higher than the difference within the groups, therefore there is a significant difference in the mean elongation of shoots on different treatments for both elongation (F=89.5, sig. 0.00) and rooting (F=3.00, sig=0.00). Different letters indicate statistically significant groups.

Table 3.4: Effect of GA_3 on rooting of cuttings versus starting length. Successful rooting was measured after 3 weeks in culture, as indicated by the percentage of propagated shoots that exhibited root induction.

	Shoot length (cm)						
GA concentration (mg L^{-1})	0	0.5	1.0	2.0	3.0	4.0	5.0
0	13.3 ± 13.3^{a}	86.,6 ± 13.3^{b}	66.6 ± 33.3^{ab}	100.0 ± 0.00^{b}	100.0 ± 0.00^{b}	100.0 ± 0.00^{b}	100.0 ± 0.00^{b}
0.1	26.7 ± 17.6^{a}	86.7 ± 13.3^{b}	100.0 ± 0.00^{b}	100.0 ± 0.00^{b}	90.0 ± 8.2^{b}	100.0 ± 0.00^{b}	100.0 ± 0.00^{b}
1.0	26.7 ± 26.7^{ab}	93.3 ± 6.6^{b}	100.0 ± 0.00^{b}	100.0 ± 0.00^{b}	100.0 ± 0.00^{b}	100.0 ± 0.00^{b}	93.3 ± 6.7^{b}
3.0	46.7 ± 24.0^{a}	70.0 ± 8.2^{b}	100.0 ± 0.00^{b}	100.0 ± 0.00^{b}	100.0 ± 0.00^{b}	93.3 ± 6.7^{b}	100.0 ± 0.00^{b}
5.0	33.3 ± 33.3^{ab}	73.3 ± 13.3^{b}	86.7 ± 13.3^{b}	86.7 ± 13.3^{b}	90.0 ± 8.2^{b}	100.0 ± 0.00^{b}	100.0 ± 0.00^{b}

Multivariate ANOVA values indicate that the mean (± SE) difference between the groups was higher than the difference within the groups, therefore there is a significant difference in the mean elongation of shoots on different treatments for both elongation (F=89.5, sig. 0.00) and rooting (F=3.00, sig=0.00). Different letters indicate statistically significant groups.

3.3 Discussion

The development of an efficient method of propagating *B. sinuspersici* through cuttings is a valuable tool in providing a ready supply of plant material for research. The effects of different auxins on the induction of roots has also provided valuable information on rooting cuttings as well as providing information about regenerating roots from *in vitro*-derived shoots.

Optimizing the amount of Murashige and Skoog basal medium required for root induction aids in the development of a rooting protocol. Murashige and Skoog basal medium is comprised of many different nutrients initially optimized to the *in vitro*

regeneration of tobacco. It is useful for the growth of most plant species, but its formulation is prepared in very high concentrations, particularly of nitrogen, and may be desired in as much as a 10-fold dilution by a particular species and explant type (Huang and Murashige, 1977). Thus, lowering the strength of MS medium is often used *in vitro* culture for rooting of cuttings, as reported in 'Delicious' apple, *Malus domestica* (Zimmerman, 1984) and *Ophiorrhiza prostrata*, an anti-cancer drug producing plant (Martin et al., 2008). These data further confirm that the exogenous macro and micronutrients supplied by MS medium are sufficient to induce root formation in *B. sinuspersici*. Half-strength MS medium was determined to be optimal for rooting of *B. sinuspersici in vitro*.

Auxins are phytohormones critical to root growth and development. Plants use auxin gradients to promote cell division, determine cell fate, and establish environmental controls that regulate overall root development through multiple biosynthetic pathways (Overvoorde et al., 2010). Without the presence of exogenous auxins, *in vitro* root formation relies on endogenous auxin synthesized in the shoot apex and transported downwards to create an auxin gradient required for root induction (Grieneisen et al., 2007). This established gradient of auxin allows cells to maintain information about their growth and development past the initial signals that caused cell differentiation, while changes in the auxin gradient allows the plant to control its development (Grieneisen et al., 2007). These developmental changes can be induced artificially to promote root cell differentiation by adding exogenous auxins. Although root induction of cuttings was effective using hormone-free media (Fig. 3.2A), auxins may induce healthier, longer, and more numerous adventitious roots. Moreover, examining the effect of auxins on the

development of roots in *B. sinuspersici* provides valuable information about which auxins to use when rooting *in vitro*-derived shoots.

Each of the auxins examined (2,4-D, IAA, IBA, and NAA) affected the *in vitro* induced roots differently. Medium supplemented with 1mg L^{-1} IBA produced the longest roots and facilitated a high survival rate among plants after transplantation to soil. 2,4-D, IAA and NAA were determined to be poor rooting hormones for *B. sinuspersici* due to the formation of short, unhealthy roots and low survival rates when transplanted to soil. Studies comparing the effects all four examined auxins favoured IBA as a root induction agent in both *Plumbago zeylanica* (Saxena et al., 2000) and *Eucalyptus* spp. (Fogaça and Fett-Neto, 2005). Root induction using IBA were also observed in African Blackwood, *Dalbergia melanoxylon* (Amri et al., 2009), Indian horsechestnut, *Aesculus indica* (Majeed et al., 2009), and ponytail palm, *Nolina recurvata* (Bettaieb, 2008). High concentrations of auxins are required for root initiation, but may inhibit development (De Klerk et al., 1999). It has been suggested that IBA is the most efficient of the common auxins at inducing root growth because its longer side chain length makes it more difficult for plants to oxidize (Fawcett et al., 1960). The ability of IBA to be metabolized to IAA may create a slow release to lower the concentration and allow root development to progress gradually, making it more suited to root development rather than root intiation compared to other auxins (Fogaça and Fett-Neto, 2005). However, NAA is even more stable, and thus may be inhibitory to development due to a constantly high concentration. These results suggest that IBA has an impact on root growth by promoting and accelerating cell differentiation to form roots and increasing of rate of root growth, while 2,4-D, IAA, and NAA inhibit root development and elongation. This observation is

consistent with our results using *B. sinuspersici*, where the long roots with a high survival rate upon transplanting to soil were initiated on cuttings cultured on medium supplemented with IBA, while cuttings cultured on medium containing 2,4-D, IAA, or NAA were less successful at producing successful rooted cuttings.

Results from cuttings of various lengths cultured on GA_3 indicate that cuttings of at least two to three centimeters will provide sufficient elongation to consistently form roots. While the objective of this experiment was to determine the length of *in vitro*-derived shoots required for the induction of roots, it must be taken into consideration that cuttings and *in vitro*-derived shoots may respond differently to hormones. This is likely due to hyperhydricity stress in *in vitro* propagated shoots (Kevers et al., 2004).

Although most rooting experiments were performed on medium solidified with agar, the use of Gelzan, a low-acyl gellan gum, was used as a substitute in one experiment (data not shown). Gelzan is more pure than agar and when solidified forms an almost transparent gel. Even at 0.3 %, the media was too hard for *B. sinuspersici* roots to grow through the matrix, and the high amount of light infusing through the transparent media may have inhibited root formation. Due to the high purity of the gelling agent, the media did not solidify in the condition containing 0 MS, thereby removing the control condition from the experiment. For these reasons, Gelzan was shown to be an unsuitable gelling agent for *B. sinuspersici* rooting media.

Cytological studies of vegetatively propagated plants show 2n=18 chromosomes present in *in vitro*-derived plants. The chromosome count corresponds with previous cytological studies of seed-derived plants (Akhani et al., 2005), and suggests that poly- or aneuploidy is not present in plants generated from cuttings.

In conclusion, the development of an efficient method of propagating *B. sinuspersici* through cuttings is essential in maintaining a supply of plant material for research. The effects of different auxins on the induction of roots have provided valuable insights into regenerating roots from *in vitro*-derived shoots. Rooting of cuttings may be optimally induced on hormone-free, half-strength MS medium, while IBA is the most efficient auxin to induce roots in *B. sinuspersici* and may be used to facilitate root development *in vitro*-derived shoots.

Chapter 4

In vitro propagation of *Bienertia sinuspersici* from axillary buds via direct organogenesis

4.1 Introduction

Direct organogenesis provides a means of whole plant regeneration *in vitro*. In contrast to indirect organogenesis, direct organogenesis is the generation of plant organs from differentiated cells, bypassing the intermediary steps of undifferentiated callus formation and shoot induction. Direct organogenesis allows for faster and more successful regeneration of plants *in vitro* (Hicks, 1980). Prior to this work, there were no documented reports of *in vitro* propagation of *B. sinuspersici.* Thus many combinations of phytohormones were tested to determine their effects on organogenesis of *B. sinuspersici*.

Apical and axillary buds from mature plants were used as initial shoot explants. Shoots were replicated on media containing various concentrations of BAP, a cytokinin commonly used to propagate shoots (Letham, 1967). Elongation is necessary to separate the shoot tip auxins from the region of root formation, as auxins are synthesized in the apical shoot and transported basipetally to the roots, resulting in a gradient that controls development (Cline, 1991). Optimization of culture conditions for shoot multiplication, elongation, and rooting provides a reproducible procedure for micropropagation via direct organogenesis of *B. sinuspersici*.

4.2 Results

4.2.1 Shoot Induction

The addition of BAP to the growth medium resulted in shoot induction. After three weeks on MS medium supplemented with BAP, axillary bud explants divided and formed new shoots, whereas explants cultured on the hormone-free media showed very little response with only 2% of shoots dividing to form additional shoots (Table 4.1; Fig. 4.1B). When BAP was added to the growth medium, between 48 and 66% of the cultured shoots formed additional shoots in all tested concentrations (Table 4.1).

Table 4.1: Effect of BAP on *de novo* formation of apical and axillary buds of *B. sinuspersici.*

Concentration of BAP (mg L^{-1})	Percentage response	Number of new shoots/explant
0	2 ± 2[a]	1.0 ± 0.02
0.5	48 ± 11[b]	1.9 ± 0.3
1.0	65 ± 7[b]	2.1 ± 0.2
2.0	58 ± 12[b]	2.0 ± 0.3
4.0	62 ± 9[b]	2.2 ± 0.3
6.0	50 ± 15[b]	1.9 ± 0.3
8.0	62 ± 11[b]	2.2 ± 0.2
10.0	55 ± 2[b]	1.9 ± 0.1
15.0	58 ± 9[b]	2.2 ± 0.2
20.0	66 ± 12[b]	1.9 ± 0.2

Data (means ± SE) for shoot induction from axillary buds. Different letters in the column represent statistically distinct groups of treatments with respect to numbers of responsive shoots. One-way ANOVA tests indicate that the mean number of responsive shoots cultured on all treatment concentrations of BAP are statistically significant from those cultured on a hormone-free medium (F=3.25, sig=0.004, n=6). The mean numbers of shoots formed per explant do not significantly differ from one another with regards to hormone treatment (F=1.09, sig=0.386, n=6)

In general, each tested concentration of BAP generated two shoots for every explant plated, while on the hormone-free control, there was only one shoot per explant. Statistically, there is a significant difference between the hormone-free control and each of the BAP treatments, but no significant difference within the BAP treatments. *In vitro*-derived shoots were proliferated on medium containing 1 mg L^{-1} BAP, as higher BAP concentrations did not cause substantial increase in number of shoots (Table 4.1; Fig. 4.1C).

4.2.2 Elongation

BAP alone was sufficient for shoot bud induction and proliferation, however, the BAP-derived shoots failed to elongate on the same medium, or even after transfer to hormone-free MS medium (Table 4.2). Hence, it was necessary to develop a suitable media for elongation of *in vitro*-derived shoots of *B. sinuspersici*. To determine the effect of phytohormones on the elongation of shoots, *in vitro* replicated shoots were cultured on various media. Measurements of shoot length (in millimeters) were taken at the time of plating and again after five weeks. These values were subtracted to determine how much the shoot elongated during the five-week period. Lowering the concentration of BAP alone or in conjunction with a small amount of an auxin has been effective at inducing elongation in many plant species (Thakur et al., 2008; Rashid et al., 2010; Singh et al., 2009). In *B. sinuspersici*, on average, each shoot only elongated a few millimeters. In the most successful treatment, 0.001 mg L^{-1} BAP and 0.0005 mg L^{-1} NAA, an average of 3.8 mm of elongation was observed after five weeks. None of the treatment conditions were statistically different from the hormone-free control (Table 4.2). As demonstrated

in chapter 3, shoots must be at least two to three centimeters long in order to produce roots. Lowering the concentration of BAP alone was ineffective in promoting shoot elongation in *B. sinuspersici*.

Table 4.2: Effect of BAP, IAA, and NAA on elongation of shoots of *B. sinuspersici*.

Hormone Concentrations (mg L^{-1})	Average Elongation (mm)
0	1.1 ± 0.3
0.001 BAP	2.4 ± 0.7
0.001 BAP, 0.0005 NAA	3.8 ± 1.0
0.001 BAP, 0.0005 IAA	1.7 ± 0.4
0.001 BAP, 0.0005 NAA, 0.0005 IAA	1.4 ± 0.3
0.002 BAP	1.1 ± 0.3
0.002 BAP, 0.001 NAA	1.6 ± 0.2
0.002 BAP, 0.001 IAA	1.9 ± 0.4
0.002 BAP, 0.001 NAA, 0.001 IAA	1.8 ± 0.3
0.005 BAP	2.0 ± 0.9
0.005 BAP, 0.0025 NAA	1.1 ± 0.6
0.005 BAP, 0.0025 IAA	0.8 ± 0.5
0.005 BAP, 0.0025 NAA, 0.0025 IAA	2.2 ± 0.6

One-way ANOVA tests indicate that the mean elongation values do not differ significantly from one another at the 95% level (F=1.56, sig=0.120, n=9).

A combination of GA_3 and TDZ was used in efforts to stimulate shoot elongation. Overall, shoots that were cultured on media containing GA_3 and TDZ showed more elongation than those cultured on a lower concentration of BAP. The highest shoot elongation was observed (8.1 mm) in the treatment containing 0.000025 mg L^{-1} TDZ and 0.000375 mg L^{-1} GA_3 (Table 4.3).

Table 4.3: Effect of GA3 and TDZ on elongation of shoots of *B. sinuspersici*.

Hormone Concentrations (mg L^{-1})	Average Elongation (mm)
0	1.7 ± 0.3^{a}
0.000025 TDZ, 0.000375 GA_3	8.1 ± 0.6^{b}
0.00005 TDZ, 0.000375 GA_3	6.0 ± 0.6^{c}
0.000075 TDZ, 0.000375 GA_3	4.0 ± 0.6^{d}
0.000025 TDZ, 0.00375 GA_3	6.1 ± 0.6^{c}
0.00005 TDZ, 0.00375 GA_3	4.6 ± 0.5^{d}
0.000075 TDZ, 0.00375 GA_3	4.7 ± 0.7^{d}
0.000025 TDZ, 0.01125 GA_3	4.6 ± 0.7^{d}
0.00005 TDZ, 0.01125 GA_3	4.1 ± 0.6^{d}
0.000075 TDZ, 0.01125 GA_3	5.1 ± 0.6^{d}

One-way ANOVA tests indicate that the mean elongation values do differ significantly from one another at the 95% level (F=6.62, sig=0.00, n=6). Different letters represent statistically different elongation values (mean ± SE).

With repeated subcultures, most shoots showed hyperhydricity, a condition where the stress of *in vitro* conditions impairs plant growth and development (Kevers et al., 2004). Therefore, new shoots were excised, multiplied on BAP, and subcultured to fresh medium in order to improve their response to tissue culture conditions. When freshly multiplied shoots were cultured on media containing GA_3 alone, elongation increased substantially.

In vitro-derived shoots cultured on hormone-free medium elongated on average 7.8 mm, whereas those on medium containing GA_3 consistently elongated up to 12 mm (Fig. 4.1D). This response is likely due to the use of younger shoots. While the average elongation of 12 mm was achieved in GA_3-treated shoots, elongation up to 20 mm was frequently observed in some shoots. Each of the GA_3 treatment conditions was

statistically different from the hormone-free treatment, but none were distinct from each other (Table 4.4).

Table 4.4. Effect of GA3 on the elongation of BAP-derived shoots of *B. sinuspersici.*

Hormone Concentrations (mg L^{-1})	Average Elongation (mm)
0	7.8 ± 0.6^{a}
0.1 GA_3	12.5 ± 0.7^{b}
1.0 GA_3	12.4 ± 0.7^{b}
3.0 GA_3	10.9 ± 0.7^{b}
5.0 GA_3	12.1 ± 0.7^{b}

One-way ANOVA tests indicate that the mean elongation value (± SE) for the hormone-free treatment differs significantly from all the treatments containing GA_3, however the hormone-containing treatments do not differ significantly from each other (F=38.96, sig=0.00, n=6). Different letters represent statistically different elongation values according to Tukey's test.

4.2.3 Rooting

Consistently, 25-30% of *in vitro*-derived shoots developed roots while cultured on hormone-free media, or on media supplemented with low concentrations of BAP for elongation or multiplication (Fig. 4.1E and F). Few of these rooted shoots were transferred to soil (Fig. 4.1G) but many failed to survive the extended period of acclimatization in the green house. For root induction, elongated shoots were carefully dissected to remove any hyperhydrated or callus tissue present at the cut end that could lead to root formation directly from callus tissue, thus lacking vascular connections to the plantlet. Previous studies using cuttings of *B. sinuspersici* showed that IBA was the most

effective auxin at inducing root formation (Chapter 3). Therefore, IBA was used in experiments involving *in vitro*-derived shoots. Elongated shoots of at least 1.5 cm were transferred to media containing different concentrations (0.1, 1.0, 3.0, and 5.0 mg L^{-1}) of IBA. After 6 weeks, none of the *in vitro*-derived shoots developed roots in any of the seven replicates (each containing nine shoots). The cut ends of the stems often became necrotic and sometimes formed small amounts of callus tissue. In addition, varying the culturing conditions such as reducing the light intensity of the growth chamber, adding 0.1% activated charcoal to the media, and wrapping the base of Magenta jars with aluminum foil to prevent light from inhibiting root growth were attempted without success. Another experiment was attempted to induce root formation *ex vitro* where the cut ends of elongated shoots were dipped in 0.1% IBA powder and placed into the dampened foam base (data not shown). After 3 weeks, the elongated shoots had died and no roots had formed.

As an alternative way to complete whole-plant regeneration of *in vitro*-derived shoots, micrografting was used to combine elongated *in vitro*-derived shoots with the root system formed by cuttings. Both the shoot and cutting were removed from *in vitro* conditions and cut such that the stems were joined with a ‘v’ shaped wedge and secured using aluminum foil. To date none of the grafted plants survived longer than 4 weeks after transplanting to soil (Fig. 4.1H).

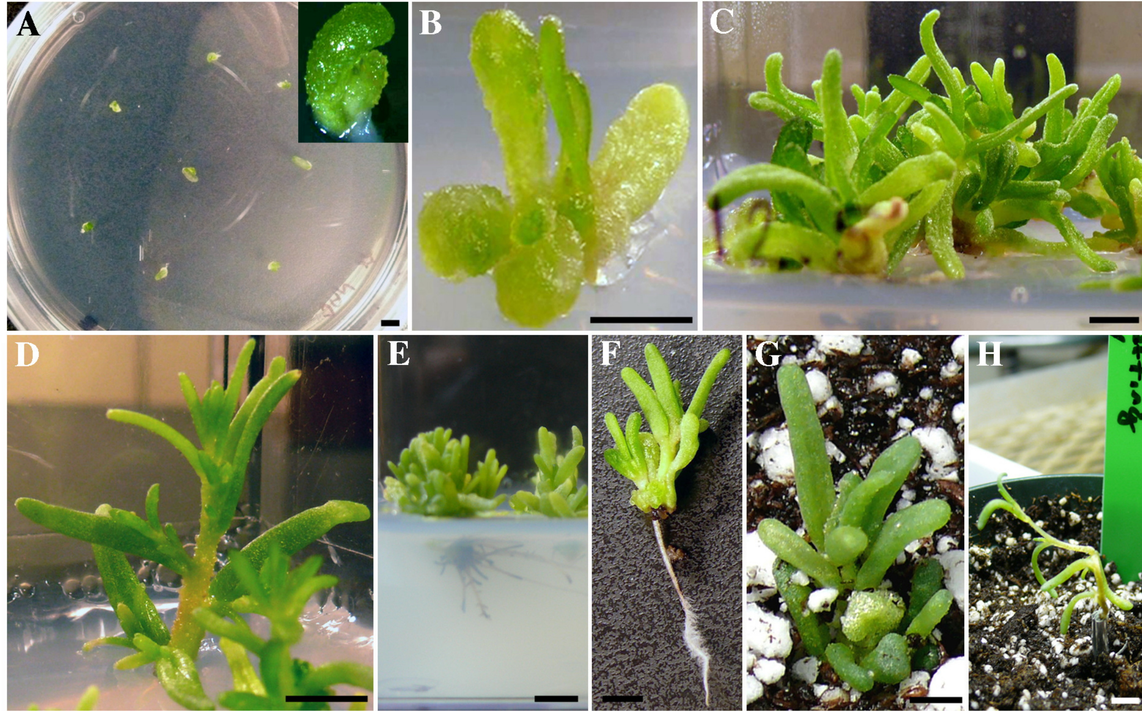

Figure 4.1 Effect of 6-benzylaminopurine (BAP) on the proliferation of apical and axillary shoots of *Bienertia sinuspersici.*

(A) Apical and axillary shoots are excised and cultured on medium supplemented with BAP. (B) After three weeks, additional shoot formation is observed. (C) Multiple repeated subcultures result in many shoots. (D) Elongation of shoots is obtained when shoots are cultured on GA_3. (E and F) Rooting of *in vitro*-derived shoots occurs on elongation medium. (G) Rooted plants are transplanted to soil. (H) Elongated in vitro-derived shoot grafted onto rootstock from cutting after 3 weeks. Scale bars = 5 mm.

4.2.4 Histological analysis of anatomy of BAP-derived leaves

The general leaf anatomy of greenhouse-grown and BAP-derived plants was compared to analyze the preservation of single-cell C_4 anatomy. Cross sections of leaves from *in vitro*-derived shoots indicate that *B. sinuspersici's* unique single-cell C_4 anatomy was maintained, compared to those from leaves of plants grown in the greenhouse from seed or cuttings (Fig. 4.2). In *B. sinuspersici,* the typical leaf anatomy consists of one to

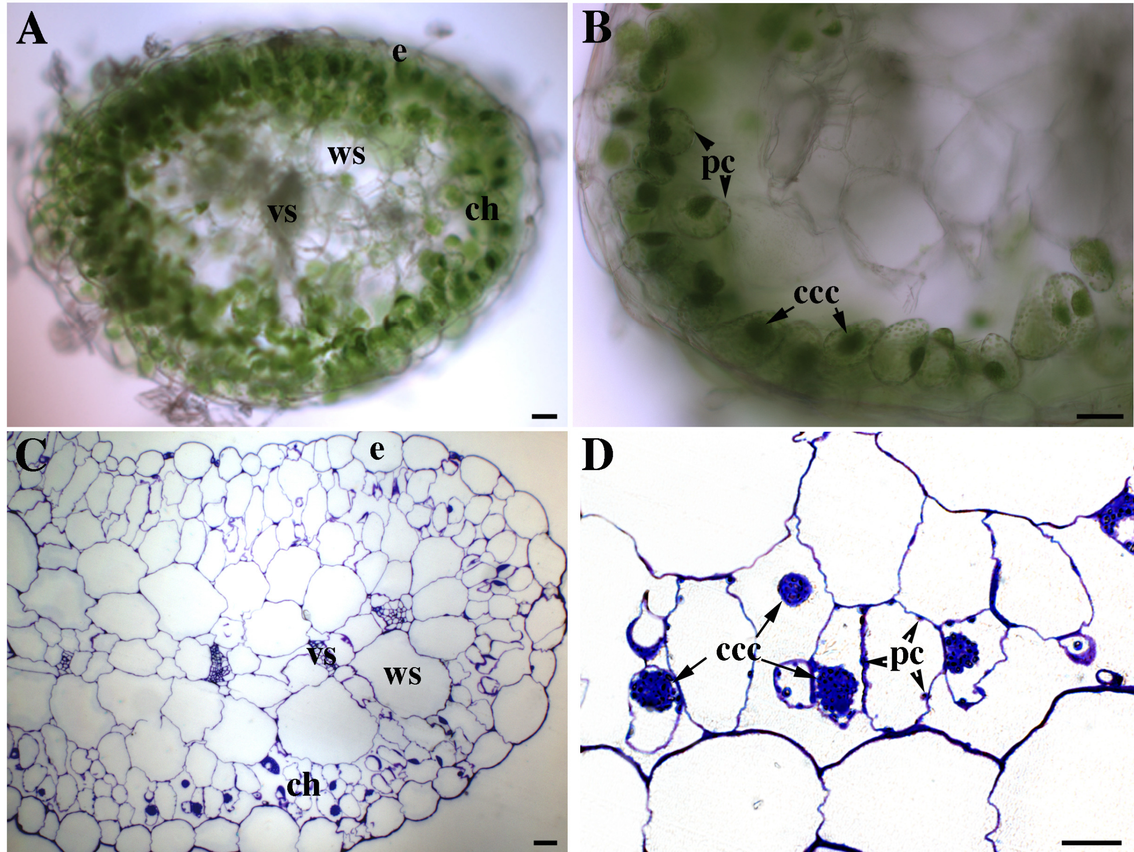

Figure 4.2 Light micrographs of leaf cross sections of BAP-derived mature leaves of *Bienertia sinuspersici* showing the general anatomy.

Cross sections were prepared by hand from fresh *in vitro*-derived mature leaves (A and B) and by microtome from LR White embedded leaf samples (C and D). Chlorenchyma cells (ch) have well-defined peripheral cytoplasmic compartment (pc) and central cytosplasmic compartment (ccc). Scale bars = 50 µm.

two layers of chlorenchyma cells with chloroplasts containing a distinctive ball-like structure suspended in the centre of the cell, large vacuolated water-storage cells, and vascular tissues (Fig. 1.1). In leaves of *in vitro*-derived shoots, the prominent single-cell C_4 feature, the ball-like cluster of chloroplasts in the central cytoplasmic compartment (CCC), was observed in most chlorenchyma cells (Fig. 4.2C and D). However, the CCC

was often positioned to one side of the cell instead of being suspended in the center, as was observed in leaves of plants grown in the green house or a growth chamber. Moreover, free-hand sections of leaves from *in vitro*-derived shoots confirmed a similar cellular structure in chlorenchyma cells showing that the distinctive organization of the central cytoplasmic compartment was shifted to the side or to the bottom of the cell (Fig. 4.2A and B).

4.2.5 Immunoblot analysis of BAP-derived shoots

Immunoblot blot analysis was performed to determine whether key C_4 enzymes were present in leaves of *B. sinuspersici* obtained via the direct organogenesis protocol. Total soluble protein was extracted from leaf tissues of BAP-derived shoots and resolved by SDS-PAGE. The levels of photosynthetic enzymes were determined by Western blot analysis using specific antibodies raised against the large subunit of Rubisco, PEPC, PPDK and NAD-ME polypeptides. Controls used for this experiment were green house grown *B. sinuspersici* and *Suaeda linifolia*, a C_3 member of the family Chenopodiaceae.

Figure 4.3 shows the Western blot analysis results of proteins prepared from leaf tissues of BAP-derived shoots that were maintained in multiplication and elongation media. Rubisco large subunit polypeptides were detected in all extracts, with higher levels in the C_3 species, *S. linifolia* (Fig. 4.3, lower panel). Signature C_4 enzymes PEPC, PPDK, and NAD-ME were detected in the extract of the positive control, greenhouse grown *B. sinuspersici* (Fig. 4.3, lane 2), as well as in samples of the BAP-derived shoots maintained on multiplication and elongation media (Fig. 4.3, lanes 3 and 4). These C_4

enzymes were not detected in the extract of *S. linifolia*, a C_3 species closely related to *B. sinuspersici* but lacking C_4 photosynthesis (Fig. 4.3, lane 1).

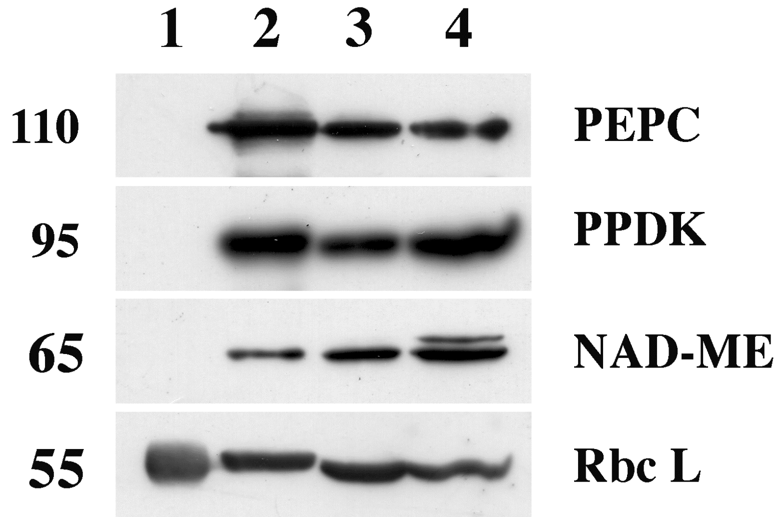

Figure 4.3 Immunoblot analysis of proteins extracted from *in vitro*-derived leaves.

Total proteins from mature leaves of greenhouse grown (1) *Suaeda linifolia*, (2) mature leaves of greenhouse grown *B. sinuspersici*, (3) leaves of BAP-derived shoots in multiplication media, and (4) leaves of BAP-derived shoots in elongation media were separated on 10% SDS-PAGE, blotted to nitrocellulose membrane and probed with anti-Rbc L antibody, anti-*Amaranthus* NAD-ME, anti-maize PPDK, or anti-maize PEPC.

4.3 Discussion

In this chapter, experiments are described where apical and axillary buds from mature greenhouse- or growth chamber- grown plants were cultured on media containing various concentrations of BAP in attempts to establish a rapid and efficient *in vitro* propagation protocol for *B. sinuspersici*. The percentage of responsive shoots to BAP was high (48-66%, Table 4.1) regardless of the concentrations used. On MS medium

without BAP, a low percentage (2%) of responsive explants was observed. These results indicate that BAP plays a role in shoot propagation in *B. sinusperisici* and are in agreement with reports showing the effectiveness of BAP at inducing shoot multiplication in many plants including grass, *Cymbopogan nudus* (Chan et al. 2005), wild service tree, *Sorbus torminalis* (Mala et al. 2009), bamboo, *Guadua angustifolia* (Jiménez et al, 2006), a medicinal herb, *Andrographis paniculata* (Purkayastha et al, 2008), and evergreen shrub, *Ilex glabra* (Sun et al. 2010). The generation of axillary shoots *in vitro* may be induced either by cytokinins causing periclinal divisions of cells in the existing shoot apical meristem (SAM) or by signaling cells to regenerate new SAMs (Kerstetter and Hake, 1997). Cytokinins, including BAP, induce the formation of SAMs during shoot organogenesis (Sugiyama, 1999). Excised axillary buds treated with exogenous cytokinins show increased rates of cell division, elongation, and DNA replication (Rubenstein and Nagao, 1976).

In *B. sinuspersici,* BAP proved to be effective at inducing shoot multiplication but not for shoot elongation (Table 4.2). Similar observations were made in douglas fir shoot cultures where an increase in BAP concentrations resulted in the development of minute, stunted shoots (Winston and Verhagan, 1977) and abnormal growth in the ornamental plant, *Spathiphyllum floribundum* (Werbrouck et al. 1995, 1996). Lowering the concentration of BAP used for shoot induction either alone or in conjunction with a small amount of an auxin has been effective at inducing elongation in many plant species including pears (Thakur et al., 2008), tomatoes (Rashid et al., 2010), and *Jatropha curcas* – an important biodiesel plant (Singh et al., 2009). However, regenerated shoots of *B. sinuspersici* demonstrated little elongation on media with low BAP concentrations or in

combination with an auxin. This suggests that BAP inhibits shoot elongation in *B. sinuspersici*. An inhibitory effect of BAP on shoot elongation has also been observed in many micropropagation protocols (Iriondo et al. 1995; Ault 1994; Brassard et al. 1996; Figueiredo et al. 2001). Moreover, low concentrations of BAP are often recommended to replicate shoots *in vitro* because of interactions between residual hormones will affect micropropagation success during elongation and rooting (Malá et al., 2009).

In this study, GA_3 was the most effective hormone at promoting shoot elongation, indicating that GA_3 may play an important role in the elongation of *B. sinuspersici*. GA_3 has been routinely used in many different tissue culture protocols to successfully induce shoot elongation in dwarf dogwood, *Cornus canadensis* (Feng et al, 2009), *Cannabis sativa* (Lata et al., 2009), and an important medicinal creeper vine, *Aristolochia indica* (Manjula et al., 1997). GA_3 is particularly useful at inducing elongation in species recalcitrant to normal elongation *in vitro* (Phinney, 1984). GA_3 has been used simultaneously with cytokinins to both induce shoot multiplication and elongation in jojoba, *Simmondsia chinensis* (Mohasseb et al., 2009) and rose, *Rosa hybrida* (Davies, 1980), while increasing shoot multiplication yield (Pati et al., 2006). In *B. sinuspersici,* 0.1 mg L^{-1} of GA_3 is recommended for shoot elongation (Table 4.4, Fig. 4.1D). Due to the risk of hyperhydricity in shoots that had been repeatedly subcultured, it is recommended that shoots be transferred to elongation media within two or three months of development to maximize shoot elongation (Kevers et al., 2003).

Although many shoots spontaneously formed roots during the shoot elongation step, elongated shoots did not root when placed on media containing IBA or when cultured *ex vitro* with IBA rooting powder. Difficulties with root induction have been

observed in micropropagtion techniques for many tree species (Mala et al. 2009). The poor rooting ability of these tree species has been attributed to the accumulation of metabolic products of BAP such as BAP9G (Werbrouck et al. 1995, 1996). Thus, the lack of root formation in the elongated *in vitro* shoots of *B. sinuspersici* may also be due to increased levels of BAP conjugates. The accumulation of BAP conjugates can be minimized by excising the base ends of elongated shoots to remove hyperhydrated tissue prior to plating on rooting medium. Regardless, many rooted shoots did not survive transplantation. This could be due to roots emerging from hyperhydrated tissue at the base of the stem that did not have direct vascular connections to the aerial part of the plant. Thus, root induction in *B. sinuspersici* may also have been inhibited by hyperhydricity. In *Scrophularia yoshimurae*, removing the parafilm from culture vessels to release the high concentrations of CO_2 and ethylene, reduce humidity, and improve gas exchange in the culture chamber resulted in reduced hyperhydricity (Lai et al., 2005).

Less conventional method for *in vitro* propagation such as micrografting appears to be a promising method of rooting micropropagated shoots. Grafting has been successfully used in the regeneration protocol for many species that are otherwise difficult to root *in vitro,* including lentil, *Lens culinaris* (Gulati et al., 2001), avocado, *Persea americana* (Raharjo and Litz, 2001), year bean, *Phaseolus polyanthus* (Zambre et al, 2001), and grass pea, *Lathyrus sativus* (Zambre et al, 2002). In *B. sinuspersici*, preliminary experiments using elongated shoots grafted onto rootstock of cuttings suggested grafting as a promising option for completing the regeneration procedure as grafted plants remained viable for at least one month. This technique could be improved

to ensure the survival of the grafted plants by improving the conditions of early steps after the grafted unions are made.

The anatomy of *in vitro*-derived leaves revealed that the unique single-cell C_4 photosynthetic anatomy of *B. sinuspersici* indicated by its characteristic central cytoplasmic compartment is maintained (Fig. 4.2). The central compartment was shifted to the side of the cell during early stages of culturing, as indicated by it being pressed to the side of the cell. Similar repositioning of the central cytoplasmic compartment was observed in *B. sinuspersici* after one month under low light treatment, conditions that are similar to those in the tissue culture chambers (Lara et al., 2008). This is believed to be due to environmental stresses that may include light, temperature, and humidity of the *in vitro* conditions, or the effect of having plentiful sucrose and nutrients such that the plants put less effort into photosynthesis. Perhaps the central cytoplasmic compartment may become more developed after being weaned off sucrose and transplanted to soil. Altered leaf anatomy has been reported in *in vitro*-derived shoots, including increased air spaces between parenchyma and increased water storage cells (Kevers et al., 2003). Regardless of the organization and position of the central cytoplasmic compartment, immunoblot analysis showed high levels of key enzymes (PEPC, PPDK, and NAD-ME) in protein extracts of the *in vitro*-derived shoots suggesting that C_4 photosynthetic pathway is functional (Fig. 4.3).

This experiment illustrates that it is possible to culture and regenerate whole plants from an apical or axillary buds, and provides an efficient method for micropropagation, of *B. sinuspersici*.

Chapter 5

In vitro regeneration of *Bienertia sinuspersici* via indirect organogenesis

5.1 Introduction

The discovery of single-cell C_4 photosynthesis has offered new perspectives for introducing a carbon concentrating mechanism in C_3 crops without the development of Kranz anatomy. Although genetic transformation is a tool that can be applied to achieve this biotechnological endeavor, production of transgenic plants is often limited by lack of rapid and efficient regeneration protocols. Developing protocols for efficient genetic transformation of *Bienertia sinuspersici* is important to further understand the molecular basis of single-cell C_4 photosynthesis and to provide novel solutions for enhancing carbon assimilation in C_3 crops. To the best of our knowledge, a genetic transformation protocol is lacking for *B. sinuspersici* thus far. Currently, only transient transformation protocols using PEG-mediated transformation of protoplasts and biolistic bombardment of leaves are routinely employed to introduce genes of interest and examine their expression in cells of *B. sinuspersici* (Lung et al., unpublished, Chuong et al., 2006). Therefore, establishment of callus cultures that support the regeneration of plants is required to facilitate the development of a transformation protocol for *B. sinuspersici.*

Indirect organogenesis provides a means of dedifferentiating plant tissue into callus, allowing for transformation and selection protocols, and regenerating whole plants *in vitro* from transformed calli (Robinson and Firoozabady, 1993). Prior to this work,

there were no reports documenting the *in vitro* regeneration of *B. sinuspersici* via indirect organogenesis.

Dedifferentiation of plant material into callus tissue generally depends on the ratio of cytokinin to auxin in the plant media (Thorpe, 2007). This chapter describes the development and optimization of an efficient plant regeneration protocol from stem-derived calli of *B. sinuspersici*. Cytokinins (BAP and kinetin) and auxins (2,4-D and NAA) were examined to determine their effects on callus induction. Callus tissues were induced to form shoots only by supplementing the culture medium with TDZ. Normal single-cell C_4 photosynthetic anatomy and biochemistry, as indicated by the intracellular compartmentalization of organelles and detection of key C_4 enzymes, were confirmed by histological and immunoblot analyses, respectively, of leaves of the regenerated *B. sinuspersici* plants.

5.2 Results

5.2.1 Callus induction

Basal MS medium was supplemented with various combinations and concentrations of auxins (2,4-D and NAA) and cytokinins (BAP and kinetin) for the induction of primary callus from *B. sinuspersici* stem explants. During the initial stage, callus tissue was observed on stem explants after 2 weeks of culture in all treatments (Table 5.1; Fig. 5.1B). The percentage of explants producing callus increased markedly when auxin and cytokinin were added to the medium (Table 5.1). Most explants (60 - 100%) produced calli within 6 weeks of culture (Table 5.1).

Table 5.1: Effect of various hormones on callus formation of stem explants of *B. sinuspersici* after 2, 4 and 6 weeks in culture.

Hormone Concentrations (mg L^{-1})	Percentage of responsive explants		
	2 weeks	4 weeks	6 weeks
0	0	0	0
0.5 2,4-D + 0.5 kinetin	49.7 ± 16.0	88.5 ± 6.6	89.7 ± 4.4[a]
0.5 2,4-D + 1.0 kinetin	86.7 ± 0.0	96.7 ± 2.1	100.0 ± 0.0[a]
1.0 2,4-D + 0.5 kinetin	46.3 ± 15.1	91.4 ± 5.3	94.5 ± 3.9[a]
1.0 2,4-D + 1.0 kinetin	80.0 ± 0.0	90.0 ± 6.3	90.0 ± 6.3[ab]
2.5 2,4-D + 0.5 kinetin	53.3 ± 19.3	82.3 ± 13.9	94.8 ± 3.0[a]
2.5 2,4-D + 1.0 kinetin	52.0 ± 11.5	72.4 ± 12.4	75.6 ± 9.6[ab]
0.5 2,4-D + 0.5 BAP	53.5 ± 9.4	85.2 ± 11.5	87.0 ± 10.0[ab]
0.5 2,4-D + 1.0 BAP	44.0 ± 10.8	77.9 ± 9.7	80.0 ± 10.7[ab]
1.0 2,4-D + 0.5 BAP	83.3 ± 2.1	93.3 ± 4.2	93.3 ± 4.2[ab]
1.0 2,4-D + 1.0 BAP	41.4 ± 19.6	71.7 ± 17.4	91.0 ± 3.2[a]
2.0 2,4-D + 0.5 BAP	39.6 ± 14.8	82.6 ± 7.7	91.1 ± 6.0[a]
2.5 2,4-D + 1.0 BAP	43.1 ± 19.5	88.4 ± 5.3	96.1 ± 1.6[a]
0.5 NAA + 0.5 kinetin	21.9 ± 17.4	55.8 ± 23.2	57.7 ± 22.6[ab]
0.5 NAA + 1.0 kinetin	46.4 ± 17.9	75.8 ± 19.1	78.7 ± 19.7[ab]
1.0 NAA + 0.5 kinetin	65.7 ± 14.4	91.6 ± 2.9	91.5 ± 4.7[a]
1.0 NAA + 1.0 kinetin	86.7 ± 8.4	93.3 ± 0.0	93.3 ± 0.0[a]
0.5 NAA + 0.5 BAP	54.8 ± 23.2	55.6 ± 21.6	57.8 ± 22.6[ab]
0.5 NAA + 1.0 BAP	0.0 ± 0.0	10.0 ± 6.3	10.0 ± 6.3[c]
1.0 NAA + 0.5 BAP	66.4 ± 12.8	92.3 ± 4.9	92.3 ± 4.9[ab]
1.0 NAA + 1.0 BAP	69.2 ± 6.9	100.0 ± 0.0	100.0 ± 0.0[a]

Values (means ± SE) in each column represent the percentage of explants that formed callus from at least three independent experiments (3 plates with 12 explants per plate) after 2, 4 and 6 weeks on callus induction medium. Different letters in a column indicate a significant difference between treatments at $P \leq 0.05$ according to Tukey's test.

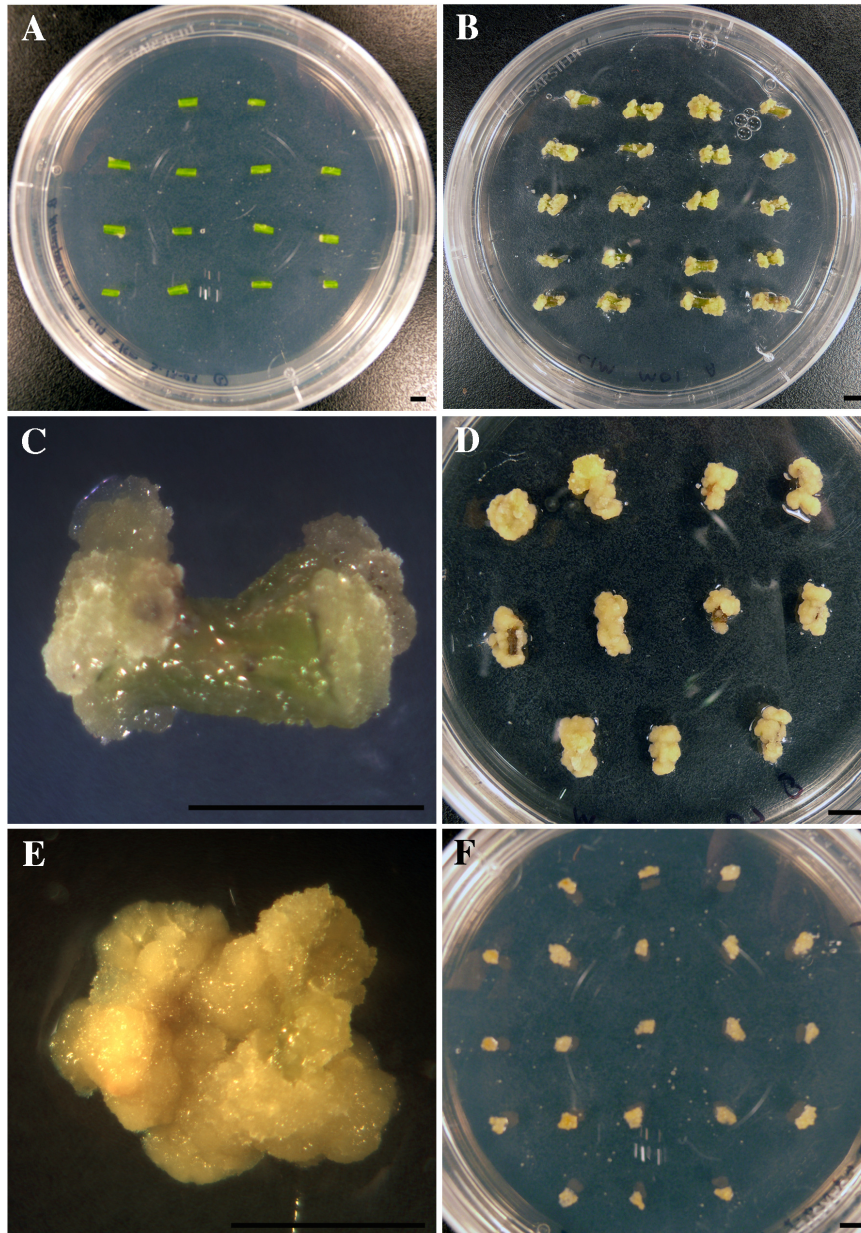

Figure 5.1 Induction of callus tissue from stem explants of *Bienertia sinuspersici* on medium supplemented with 2,4-D and kinetin.

(A) Explants on callus induction medium at day 1. (B and C) Explants after four weeks on callus induction medium exhibit prolific callus growing at the cut ends of stem explants. (D) Callus growing on stem explants after six weeks on callus induction medium. (E and F) Callus has been removed from the initial stem explants and subcultured onto fresh medium. Scale bars = 5 mm

Calli were also observed qualitatively to determine which combination of hormones produced the most prolific and healthiest calli. Generally, healthy calli should appear light yellow in colour and smooth in texture, and proliferate rapidly (Fig. 5.1D). While some treatments resulted in a high percentage of explants forming calli, only a small quantity of brown and unhealthy calli were produced. For example, explants cultured on medium containing NAA tended to produce smaller quantities and less healthy calli than those from explants cultured on medium containing 2,4-D. In the present study, it was determined that the medium supplemented with 2.5 mg L^{-1} 2,4-D, 0.5 mg L^{-1} kinetin consistently produced the highest quality calli (Fig. 5.1E). These calli were repeatedly subcultured on fresh solid medium containing the same hormone ratio and were used in the shoot induction experiments (Fig. 5.1F).

5.2.2 Shoot induction from stem-derived callus

Regenerating shoots from callus in *B. sinuspersici* is a key step in achieving whole plant transformation. Healthy calli obtained from stem explants on solid medium supplemented with 2.5 mg L^{-1} 2,4-D, 0.5 mg L^{-1} kinetin were subcultured onto solid medium supplemented with various cytokinins (kinetin, BAP, or TDZ) for the induction of shoots. Calli that were cultured on medium containing kinetin, calli quickly turned grayish-brown, stopped proliferating, and did not produced any shoots (data not shown). Calli cultured on medium containing BAP proliferated large quantities of healthy, green callus, but failed to differentiate shoots (data not shown). However, the addition of TDZ to the medium improved the shoot induction potential of the calli as many shoot primordia were observed after 6 weeks of culture (Table 5.2; Fig. 5.2A). A low

concentration of TDZ (0.001 mg L^{-1}) did not substantially increased the percentage of calli that developed shoots, whereas concentrations 0.005 mg L^{-1} or higher were more effective in promoting shoot formation with 0.1 mg L^{-1} TDZ providing the highest percentage of calli (80%) that differentiated shoots (Table 5.2). Furthermore, this treatment also induced many healthy shoots per clump of callus, making it highly effective for shoot regeneration (Fig. 5.2B).

Table 5.2: Effect of thidiazuron (TDZ) on shoot organogenesis of callus after 6 weeks in culture.

TDZ Concentration (mg L^{-1})	Percentage of responsive callus
0	0.0 ± 0.0^{a}
0.001	18.3 ± 4.9^{ab}
0.005	57.5 ± 19.8^{ab}
0.01	54.0 ± 8.1^{ab}
0.1	80.0 ± 10.9^{b}
0.25	48.0 ± 16.5^{ab}
0.50	55.0 ± 13.9^{ab}
0.75	66.0 ± 11.7^{ab}
1.0	45.0 ± 19.4^{ab}
2.0	50.0 ± 25.8^{ab}

Values (means ± SE) represent the percentage of calli that formed shoots within 6 weeks on shoot induction medium. Different letters in a column indicate a significant difference between treatments at $P \leq 0.05$ according to Tukey's test.

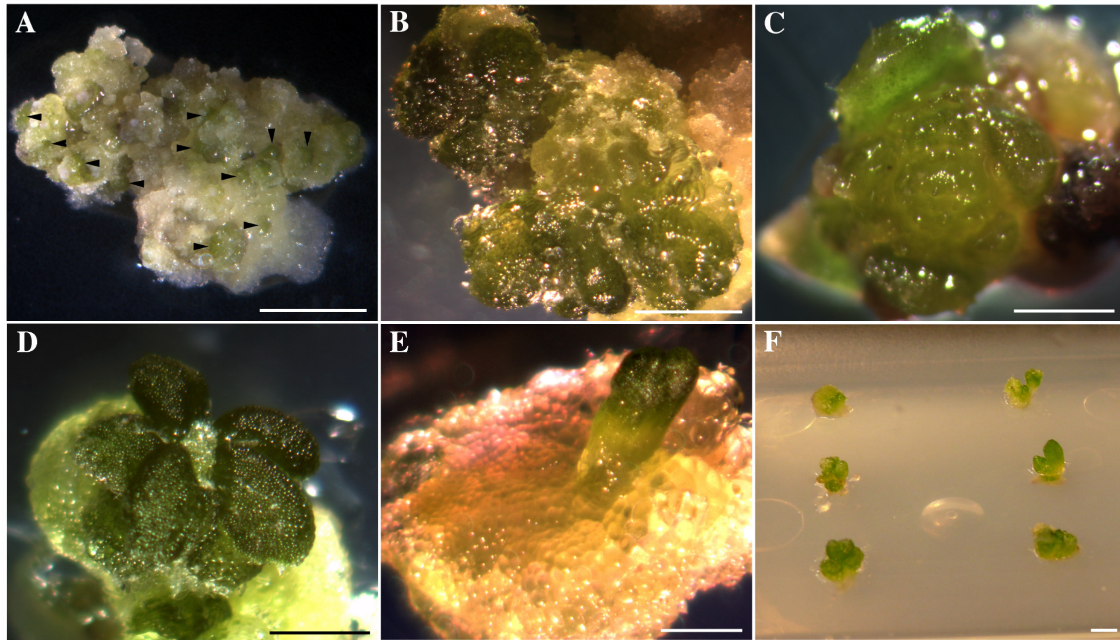

Figure 5.2 Effect of TDZ on regeneration of shoots from callus tissue in *Bienertia sinuspersici*.

(A) Initial shoot formation on responsive callus tissue (arrowheads). (B) Developing shoots emerging from callus tissue. (C) Top view of an emerging shoot. (D and E) True shoots developed via indirect organogenesis after repeated subcultures on TDZ-containing medium. (F) Excised true shoots on medium containing 2.0 mg L^{-1} 6-benzylaminopurine (BAP) for shoot replication. Scale bar = 3 mm.

In order to obtain true shoots, calli exhibiting formation of shoot primordia were subcultured onto fresh TDZ-containing medium to further develop the shoot (Fig. 5.2C-E). Any dying callus that might hinder shoot development was removed. Individual shoot primordia were isolated and plated directly on the medium surface to aid shoot formation (Fig. 5.2F). Since callus does not form vascular tissue, putting the responsive callus in as close contact with the medium as possible aids hormone uptake.

5.2.3 Multiplication and elongation of TDZ-derived shoots

After twelve weeks of subculture on shoot inducing medium, fully developed shoots were isolated and transferred to medium containing 2 mg L^{-1} BAP, which was previously determined to be effective for shoot replication in direct organogenesis experiments (Fig. 5.3B; see chapter 4). Repeated subcultures on BAP produced large quantities of TDZ-derived shoots. Proliferated shoots were plated on media containing GA_3, which was found to be most effective at elongating shoots derived via direct organogenesis (Fig. 5.3D; Chapter 4)

Table 5.3: Effect of GA_3 on the elongation of TDZ-derived shoots

GA_3 Concentrations (mg L^{-1})	Average Elongation (mm)
0	1.2 ± 0.9^a
0.1	4.5 ± 1.5^b
1.0	4.8 ± 1.3^b
3.0	3.6 ± 1.3^b
5.0	4.2 ± 1.3^b

Values (means ± SE) represent average elongation from at least three independent experiments. Different letters in a column indicate a significant difference at the 95% level ($P \leq 0.05$) according to the Tukey test. Each of the conditions containing GA_3 was statistically significant compared to the hormone-free condition, but not compared to each other ($F=11.2$, sig=0.00, n=7).

5.2.4 Induction of root in TDZ-derived shoots

It was consistently observed that 20 to 30% of TDZ-derived shoots spontaneously formed roots during subculture on shoot replication or elongation medium (Fig. 5.3C).

However, many of these rooted plantlets did not survive after transplantion to soil. Roots may have arisen directly from callus at the base of the stem and did not have vascular connections to the plant. Occasionally, elongated shoots that were cultured on low concentrations of BAP or gibberellins also exhibited root formation (Fig. 5.3E). A few of the surviving TDZ-derived plants were those that were obtained using shoots rooted on medium containing a low concentration of BAP or GA_3 (Fig. 5.3D-F). According to cytological analysis, regenerated plantlets derived through indirect organogenesis had the same chromosome number (2n=18) (Fig. 5.3F inset) as plants derived from seeds.

Elongated shoots of at least 1.5 cm were also cultured on media containing various concentrations of IBA (0.0, 0.5, 1.0, 2.5, and 5.0 mg L^{-1}) to induce adventitious root formation. Similar to the *in vitro* shoots derived via direct organogenesis experiments (Chapter 4), none of these shoots developed roots after 6 weeks. Many shoots showed moderate hyperhydricity as indicated by callus formation and necrosis of the cut ends. Alternatively, dipping the ends of elongated shoots in IBA powder and placing in dampened foam base also failed to induce roots. Grafting of TDZ-derived shoots to existing rooted stems was also performed and the resulting grafted plants were able to survive up to 4 weeks in soil.

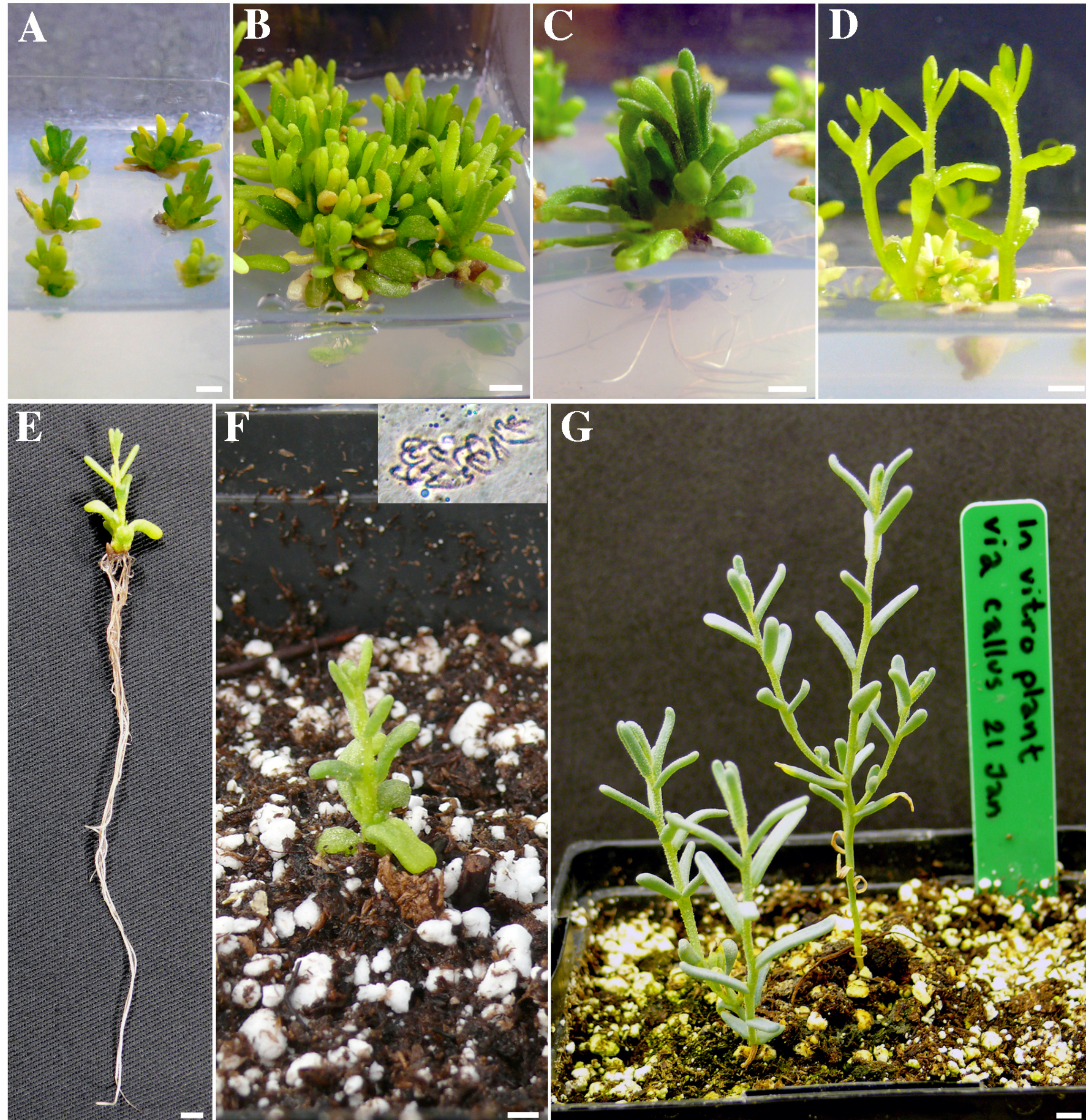

Figure 5.3 Whole-plant regeneration of *Bienertia sinuspersici* from true shoots derived via indirect organogenesis.

(A) True shoots plated on shoot replication media for mass propagation. (B) Replicated shoots after six weeks cultured on medium containing 2.0 mg L^{-1} 6-benzylaminopurine (BAP). (C) Rooted shoots on elongation medium containing GA_3 (D) Shoots elongated on medium supplemented with GA_3. (E) Elongated plantlet with long, healthy roots. (F) *In vitro*-derived plantlet transplanted to soil. (Inset in F) Root tip squash from regenerated plants showing late prophase chromosomes. (G) Regenerated plant after four months in greenhouse conditions. Scale bars = 5 mm

5.2.5 Histological analysis of leaf anatomy of TDZ-derived shoots

Histological analysis of leaves of regenerated plants was performed to determine whether *Bienertia sinuspersici*'s characteristic single-cell C_4 anatomy is maintained in the TDZ-derived plants. Leaf cross sections prepared from regenerated plants via indirect organogenesis illustrate that a central cytoplasmic compartment, indicated by a cluster of chloroplasts, was observed in most chlorenchyma cells (Fig. 5.4C). This cellular organization is seen in both young and mature leaves of *in vitro*-derived shoots (Fig. 5.4A and B). However, similar to *in vitro*-derived shoots via direct organogenesis, the central cytoplasmic compartment is not, in fact, centralized in chlorenchyma cells of young leaves, but lies against the side of the cell (data not shown). Leaf cross sections of a 4-month old regenerated plant that has been maintained under greenhouse conditions shows chlorenchyma cells with a central cytoplasmic compartment containing chloroplasts similar to those found in greenhouse grown plants (Fig. 5.4C and D).

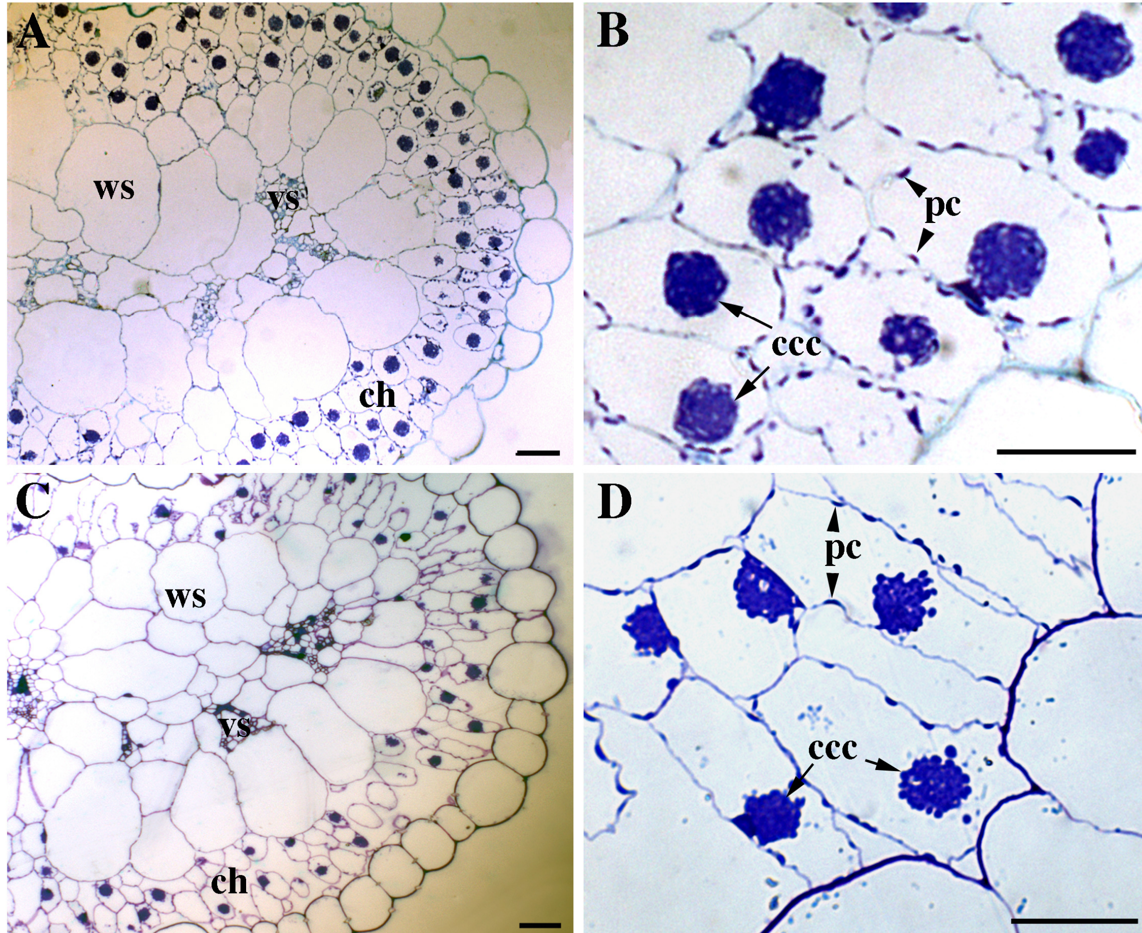

Figure 5.4 Light micrographs of leaf cross section from regenerated and greenhouse grown plants.

Cross sections compare the general anatomy of leaves of regenerated *B. sinuspersici* plants (C and D) obtained via indirect organogenesis to that of plants grown in the greenhouse (A and B). Scale bars = 50 µm. (ws= water storage cells, vs= vascular system, ch=chlorenchyma cells, pc= peripheral compartment, ccc= central cytoplasmic compartment)

5.2.6 Immunoblot analysis of TDZ regenerated plant leaves

Western blot analysis was performed to determine whether key C_4 enzymes were present in leaves of plants that were regenerated by the indirect organogenesis method.

Total soluble protein was extracted from young and mature leaves of TDZ-derived *in vitro* shoots and TDZ-regenerated plants and separated by SDS-PAGE. The levels of specific photosynthetic proteins were determined by immunoblot analysis using antisera raised against the large subunit of Rubisco, the α-subunit of NAD-ME, and the PEPC and the PPDK polypeptides. Proteins from leaves of *B. sinuspersici* plants propagated by cuttings (grown in the greenhouse and a growth chamber) were used as positive controls. In addition, *Suaeda linifolia*, a C_3 species of the Chenopodiaceae family, was used as a control (Fig. 5.5).

Figure 5.5 shows the results of the Western blot analysis of total proteins isolated from leaves of TDZ-derived shoots maintained under *in vitro* conditions, and leaves of 2-week and 4-month old regenerated plants transplanted to soil and maintained in a growth chamber under controlled conditions. Rubisco large subunit polypeptides were detected in all extracts and their amounts varied somewhat between the different tissues and species. As expected, Rubisco was present in higher quantities in the C_3 plant than in all *B. sinuspersici* leaf tissues, based on equal loading and observed band width (Fig 5.5, lane 1, lower panel). Key C_4 photosynthetic enzymes (PEPC, PPDK, and NAD-ME) were present in all *B. sinuspersici* leaf extracts, with similar amounts accumulating in mature leaves of the TDZ-regenerated plants, and those of the greenhouse- or growth chamber- grown plants (Fig. 5.5, lanes 2-8). These C_4 enzymes were not detected in *S. linifolia*, a C_3 plant (Fig. 5.5, lane 1, top three panels).

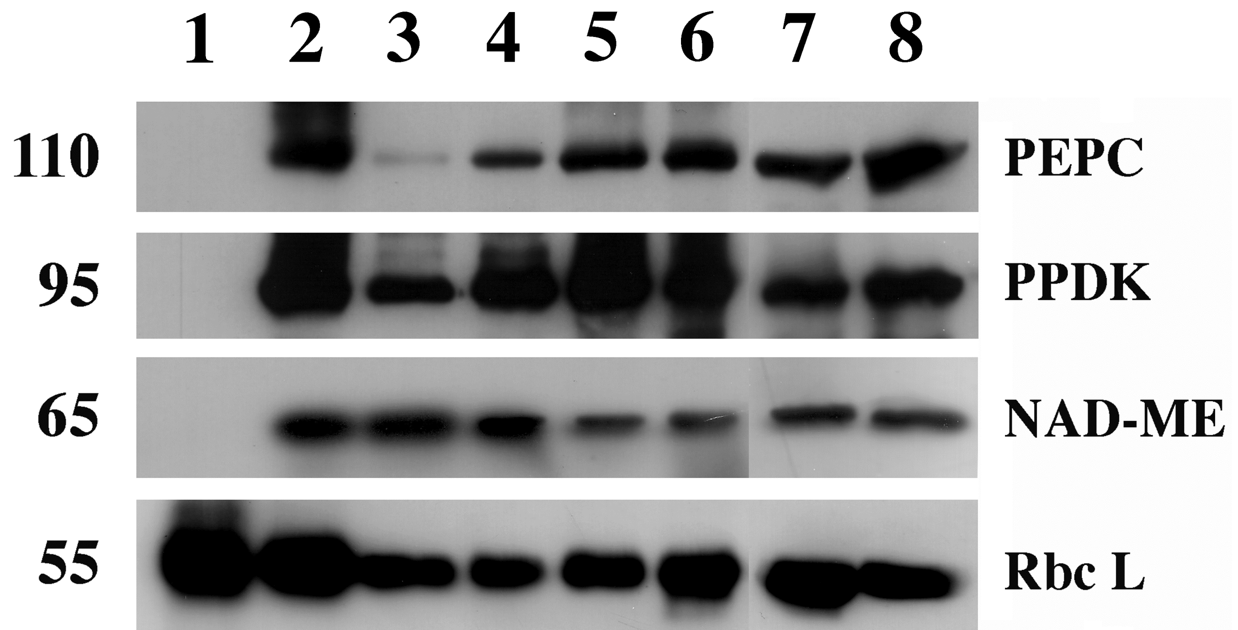

Figure 5.5. Western blot analysis of key photosynthetic enzymes in TDZ-derived *in vitro* shoots and TDZ-derived regenerated *B. sinuspersici* plants.

Total proteins from *Suaeda linifolia* (1), mature leaves of greenhouse-grown *B. sinuspersici* (2), young leaves of TDZ-derived *in vitro* shoots (3), old leaves of TDZ-derived *in vitro* shoots (4), young leaves of 2-wk old regenerated plant (5), old leaves of 2-wk old regenerated plant (6), stems of 4-month old regenerated plant (7), mature leaves of 4-month old regenerated plant (8), mature leaves of growth chamber-grown *B. sinuspersici* (9) were resolved on SDS-PAGE, blotted to PVDF and probed with anti-Rbc L, anti-*Amaranthus* NAD-ME, anti-maize PPDK, or anti-maize PEPC antibody.

5.3 Discussion

The establishment of regenerate plants via indirect organogenesis is an essential step in developing transgenic plant lines of *Bienertia sinuspersici*. This chapter describes an optimized protocol for indirect organogenesis of *B. sinuspersici,* and will provide researchers with a foundation through which to transform and regenerate plant material. Auxins and cytokinins are phytohormones that are commonly used in medium to induce

the growth of callus tissue *in vitro*, as these interact to stimulate rapid cell division (Gaspar et al., 2002). Auxins and cytokinins undergo complex interactions to influence plant growth at multiple levels, including signal transduction, gene regulation, and post-transcriptional modifications (Gaspar et al., 2002; Coenena and Loxamb, 1997). The optimum ratio of auxin:cytokinin varies between species based on their individual growth requirements. In *B. sinuspersici,* callus from stem explants can be effectively induced on medium supplemented with 2.5 mg L^{-1} 2,4-D and 0.5 mg L^{-1} kinetin. Similar results were found in studies using *Gymnema sylvestris* (Roy et al., 2008) and *Curcuma aromatica* Salisb. (Mohanty et al., 2008).

Although embryogenic calli were not observed in *B. sinuspersici*, evidence exists that 2,4-D, a synthetic auxin analog, inhibits embryo growth in some plant species. In some instances, developed embryos did not resemble those in seeds including soybeans, *Glycine max* (Lazzeri et al., 1987) curly dock, *Rumex crispus* (Monaco and Cumbo, 1972), and giant squill, *Drimiopsis kirkii* (Lan et al., 2008), although embryo growth was observed in others, including an herb native to India, *Gymnema sylvestris* (Roy et al., 2008). In the case of giant squill, increasing the concentration of 2,4-D in the growth medium promoted callus proliferation, while increasing the concentration of kinetin promoted somatic embryo formation (Lan et al, 2008). Similarly, callus proliferation was observed in explants of *B. sinuspersici* on media supplemented with 2,4-D (Fig. 5.1). This response may be due to the fact that in high concentrations, 2,4-D induces cell growth and division so rapid that the plant can no longer sustain itself and eventually dies, thus making 2,4-D one of the most potent and widely used herbicides in the world (Teixeira et al., 2007).

Successful shoot organogenesis in *B. sinuspersici* was only achieved by inducing shoots from callus tissue using medium containing TDZ, whereas other tested cytokinins such as BAP and kinetin failed to induce shoots. The highest rate of shoot induction (80%) was observed in calli cultured on medium containing 0.1 mg L^{-1} TDZ. The effect of TDZ on the induction of shoots has been observed in many species that are considered to be recalcitrant to shoot organogenesis, including *Phaseolus vulgaris* L. (Malik and Saxena, 1992), the medicinal "Cancer bush" *Sutherlandia frutescens* L. (Dewir et al., 2010), and potatoes, *Solanum tuberosum* L., (Sajid and Aftab, 2009). Although the exact mode of TDZ action has not been determined, recent evidence suggests that it can regulate endogenous cytokinin biosynthesis and/or metabolism (Mok et al., 2000). Responsive calli in *B. sinuspersici* formed many individual pre-shoots, which were repeatedly subcultured on TDZ-containing medium in order to form true shoots containing an apical meristem with surrounding leaf primordia, several true leaves, and a short stem (Fig. 5.3). Shoots were then cultured on medium containing a low concentration of BAP for multiplication.

Shoot elongation occurred optimally at a concentration of 1.0 mg L^{-1} GA_3. While gibberellic acid has been long established as a regulator of flowering, elongation, and seed germination, its mechanism and signaling pathways are not fully understood (Yamaguchi, 2008). GA_3 induces the transcription of genes that are involved in loosening the polymers within cell walls, resulting in cell elongation (Sun, 2010). It is recommended that shoot elongation be initiated as early as possible during plant regeneration, as repeated subcultures lead to hyperhydricity and stress that inhibits elongation and rooting. Shoots derived *in vitro* via indirect organogenesis did not

elongate as much over a period of 5 weeks as those derived via direct organogenesis, as described in Chapter 4. The lower rate of elongation may be caused by stress induced from the extended *in vitro* culturing conditions that these shoots were exposed to. The *in vitro* environment is very stressful to cultured explants as it is very humid, gases accumulate in the culture chamber, it lacks codependent microorganisms, and it contains unusual nutrient and hormone conditions (Kevers et al., 2004).

Rooting of *in vitro*-derived shoots spontaneously occurred on medium containing low concentrations of cytokinin or GA_3 (Fig. 5.4). Spontaneous root development has been documented in species such as eucalyptus, *Eucalyptus camaldulensis* (Dibax et al., 2010) and bamboo, *Guadua angustifolia* (Jiménez et al., 2008). Studies using *B. sinuspersici* cuttings indicated that IBA is the most efficient hormone for inducing root development (Chapter 3).

Other studies showing successful root induction in many *in vitro* cultured species using medium supplemented with IBA and activated charcoal such as *Cannabis sativa*, (Lata et al., 2008), wood-apple, *Feronia limonia* (Vyas et al., 2004) and an important Indian medicinal creeper plant, *Aristolochia indica* (Manjula et al., 1997) have been reported. However, *in vitro* rooting of elongated *B. sinuspersici* shoots on medium containing IBA failed to initiate root formation. Treating plants with IBA and culturing the shoot *ex vitro* has been successful in many species including crabapple, *Malus zumi* (Xu et al., 2007) and legume, *Pueraria tuberosa* (Rathore and Shekhawat, 2009) but failed to induce root growth in *B. sinuspersici*. Unsuccessful induction of roots may be related to high accumulation of BAP conjugates from shoot multiplication in the base of *in vitro*-derived shoots. These BAP metabolites have been proposed to inhibit root

formation of *in vitro* shoots in many plant species (Werbrouck et al. 1995, 1996). It is also possible that the stress of hyperhydrated tissue inhibited root formation, which could be corrected by improving ventilation of culture dishes (Lai et al., 2005). Alternatively, micrografting may serve as a potential solution to supplement whole plant regeneration (Fig. 5.3H).

Since genetic instability is a concern that is often associated with materials derived from *in vitro* studies, particularly with plants derived from callus tissue, cytolological analysis was performed to determine the chromosome number in the regenerated plants. The diploid chromosome number of 2n=18 observed in cells of samples of the regenerated plants, clearly indicated that there was no poly- or aneuploidy induced by phytohormones or the extended *in vitro* culturing conditions. This finding agrees with cytological analysis of vegetatively propagated plant controls described in Chapter 3 and a previous cytological study of seed-derived *B. sinuspersici* plants reporting a chromosome count of 2n=18 (Akhani et al., 2005).

Histological analysis of *in vitro*-derived shoots suggests that *B. sinuspersici*'s characteristic single-cell C_4 anatomy is present in regenerated plants. During shoot induction and replication, both young and mature leaves display evidence of a central cytoplasmic compartment. While in greenhouse grown plants the central compartment is suspended in the center of the cell, chlorenchyma cells of younger leaves or leaves maintained under *in vitro* conditions displayed poorly developed central cytoplasmic compartments often shifted to the side of the cell. This anatomical development may be associated with *in vitro* conditions permitting the plant to be less dependent on photosynthesis due to the exogenous sugar source. There are often anomalies in the

anatomy of *in vitro*-derived cell tissues due to environmental differences between the tissue culture and greenhouse environments such as temperature, humidity, and light conditions. *In vitro*-derived plants often show large intercellular spaces and hypertrophy, with less lignified vascular tissue, more spongy mesophyll cells, a reduced palisade layer, and altered arrangement of tissues (Kevers et al., 2004). Leaf sections of regenerated plants grown in greenhouse conditions for four months showed that the development of a peripheral compartment and a central cytoplasmic compartment that is centralized in chlorenchyma cells, suggesting single-cell C_4 photosynthetic anatomy is reconstituted (Fig. 5.4), and is comparable to those of greenhouse grown plants (Fig. 1.1). Furthermore, immunoblot analysis of key photosynthetic proteins in regenerated plants demonstrated that key C_4 enzymes (PEPC, PPDK, and NAD-ME) are present in high levels similar to that observed in parent stock plants that are maintained in the greenhouse or growth chamber (Fig. 5.5). These results provide further evidence that in addition to the inherited single-cell C_4 anatomy, the *in vitro*-derived plants appears to have the enzymes required for a fully functional C_4 pathway.

Overall, a reproducible protocol for whole plant regeneration from stem-derived callus tissue in *B. sinuspersici* has been developed, allowing for the establishment of genetic transformation system.

Chapter 6

Summary and General Discussion

6.1 Summary

The results from this thesis can be summarized as follows:

6.1.1 Regeneration by cuttings in B. sinuspersici

The development of a rapid propagation and regeneration method from cuttings of *B. sinuspersici* was established. The optimized procedure required less than 4 weeks from excised cuttings to transplantation to soil and involved culturing on medium containing half-strength Murashige and Skoog basal media either in hormone-free conditions or supplemented with IBA. Various auxins (IAA, IBA, NAA, and 2,4-D) were examined to determine their effect on root development and to provide insight into root induction in *in vitro* propagated shoots. IBA was determined to be the most efficient auxin at root induction in *B. sinuspersici*. Media supplemented with IBA produced the longest roots and resulted in plants that had a high survival rate after transplantation. Histological analysis of root longitudinal sections showed that roots cultured on IBA had a larger zone of cell division than those cultured on hormone-free medium. IAA, NAA, and 2,4-D were less successful at inducing root growth in *B. sinuspersici*, as shorter and less healthy roots were produced, resulting in lower survival rates.

Cuttings of various lengths were also cultured on medium containing GA_3 to determine the amount of stem elongation required for root induction. Cuttings of at least 2 – 3 cm in length had a high rate of root formation after three weeks on medium whereas

apical buds without visible internodes did not form roots. It was determined that *in vitro* propagated shoots must be elongated to at least 1 – 2 cm in order to successfully induce root growth.

6.1.2 Regeneration by direct organogenesis in B. sinuspersici

Direct organogenesis was developed in *B. sinuspersici* through apical and axillary bud cultures. The optimal condition for propagation of supernumerary axillary buds occurred on medium containing 1 mg L^{-1} BAP and repeated subcultures on media containing the same hormone concentration. *In vitro*-derived shoots were cultured on medium containing GA_3 to induce stem elongation with 0.1 mg L^{-1} GA_3 achieving the highest elongation within five weeks. In the process of whole-plant regeneration, 20-30% of plants cultured on medium containing a low concentration of GA_3 developed roots and survived transplantation to soil for several weeks. IBA added either to the MS culture medium or topically as a powder failed to induce root formation from *in vitro* propagated shoots. Micrografted shoots survived in the greenhouse for up to 4 weeks. Histological and immunoblot analyses of *in vitro*- derived leaves suggest that *B. sinuspersici*'s characteristic single-cell C_4 photosynthetic anatomy is maintained. Cross sections of leaves revealed the presence of a central cytoplasmic compartment within chlorenchyma cells. Western blot analysis detected high levels of key C_4 photosynthetic enzymes in the *in vitro* propagated leaves. The outlined procedure has proven to be an effective way to regenerate *B. sinuspersici* plants *in vitro* via direct organogenesis.

6.1.3 Regeneration by indirect organogenesis in B. sinuspersici

In vitro propagation of *B. sinuspersici* via indirect organogenesis was achieved through regeneration of whole plants from stem-derived callus tissue. Culturing stem explants of *B. sinuspersici* on MS medium supplemented with 2.5 mg L^{-1} 2,4-D and 0.5 mg L^{-1} kinetin, produced large quantities of healthy and prolific callus tissue. Calli cultured on medium supplemented with 0.1 mg L^{-1} TDZ showed the highest response of shoot regeneration after six weeks, whereas callus cultured on BAP or kinetin failed to regenerate shoots. Repeated subculturing of responsive calli onto fresh TDZ-containing medium completed shoot development. *In vitro*-derived shoots were subcultured on 2.0 mg L^{-1} BAP to generate large quantities of plant material. Regenerated shoots were transferred to medium containing GA_3 to elongate and root plantlets in order to complete whole-plant regeneration. Response of shoots to GA_3 occurred optimally at 1.0 mg L^{-1}, but may be inhibited by long culture time required to regenerate shoots from callus. 20-30% of shoots cultured on a low concentration of GA_3 spontaneously formed roots and were successfully transplanted to soil, while shoots cultured on IBA added either to culture medium or ectopically as a powder failed to induce root growth.

Histological and immunoblot analysis of *in vitro*-derived leaves suggests single-cell C_4 photosynthesis is maintained in regenerated plants, as the central cytoplasmic compartment was observed in chlorenchyma cells of leaf cross sections and key C_4 photosynthetic enzymes were found in regenerated plant leaves at various developmental stages. The regeneration of plants via indirect organogenesis and the presence of single-cell C_4 anatomy in regenerated plants indicate a successful mechanism of propagating *B. sinuspersici in vitro*. Thus, the established protocol could serve as valuable tool to

investigate the cellular and molecular regulation of single-cell C_4 photosynthesis. Dedifferentiating plant material into callus tissue and regenerating whole plants provides a means of generating transgenic plants *in vitro*.

6.2 General discussion

B. sinuspersici's unique single-cell C_4 photosynthetic mechanism has altered our understanding of how plants have evolved to improve photosynthetic efficiency. This revolutionary discovery has appealed to researchers around the world as a means of creating transgenic C_3 crops to utilize this efficient process to improve yields. As research into single-cell C_4 photosynthesis progresses, the generation of transgenic plants will provide researchers with more information about molecular and cellular mechanisms regulating this unique process.

Tissue culture provides a means of regenerating plant material. Vegetative propagation through cuttings provides researchers with large quantities of plant material of various developmental stages available for experiments. Direct and indirect organogenesis enable the regeneration of whole plants from axillary buds or undifferentiated callus tissue. Anatomical and biochemical evidence illustrating the presence of the single-cell anatomy and key C_4 photosynthetic enzymes suggest that the single-cell C_4 photosynthesis is maintained following *in vitro* propagation.

6.3 Future prospects

The ultimate objective for whole plant regeneration *in vitro* is that it provides a platform for the generation of transgenic plants. The next step is providing a protocol for stable transformation of plant material through *Agrobacterium* infiltration or use of a gene gun. Once transformed cells have been selected using an antibiotic, they may be regenerated using the established *in vitro* protocols.

Ideally, a better method of rooting plants *in vitro* could be developed. While a relatively high rooting efficiency was observed in plants cultured on GA_3, it would be useful to have a more reliable method of inducing roots. In particular, another method of rooting may ensure that the roots have vascular connections to the plant and are not the result of callus tissue forming at the base of the shoot. Since hyperhydricity is a barrier to successful root induction, perhaps now that the protocols for *in vitro* regeneration have been established, the time spent in tissue culture conditions could be substantially reduced to improve rooting success. Similarly, elongation may be improved by more efficient *in vitro* propagation, resulting in more successful rooting. Future studies may look at the accumulation of BAP metabolites in *in vitro*-derived shoots to determine whether their presence is hindering root induction, and possibly another cytokinin could be used to multiply shoots. Improving ventilation of the tissue culture chambers could reduce hyperhydricity causing a barrier to rooting success.

Additionally, alternate tissue culture protocols such as somatic embryogenesis and direct organogenesis using stem or leaf explants may provide additional options for the *in vitro* regeneration of *B. sinuspersici*.

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