IN VITRO REGENERATION AND GENETIC TRANSFORMATION OF *SESBANIA DRUMMONDII*: A MEDICINALLY AND ENVIRONMENTALLY IMPORTANT PLANT

A Thesis
Presented to
The Faculty of the Department of Biology
Western Kentucky University
Bowling Green, Kentucky

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science

By
Satish babu Cheepala

July 2003

***IN VITRO* REGENERATION AND GENETIC TRANSFORMATION OF *SESBANIA DRUMMONDII*: A MEDICINALLY AND ENVIRONMENTALLY IMPORTANT PLANT**

Date Recommended July 09, 2003

Shivendra V Bahl

Director of Thesis

Sigrid Jacobshagen

Byron Sleugh

Elmer Gray 7/29/03

Dean, Graduate Studies and Research Date

Acknowledgements

The completion of my research work and this thesis would not have been possible without the scientific expertise, guidance, and encouragement of my advisor, Dr. Shivendra V. Sahi, to whom I am immensely grateful. I am indebted to Dr. Sigrid Jacobshagen and Dr. Byron Sleugh for serving on my advisory committee despite their busy schedules. I wish to convey my special thanks to Dr. Nilesh Sharma for his guidance during my research.

I am thankful to Dr. John Andersland for his assistance with photography and microscopy. I am grateful to the Office of Graduate studies for the Graduate Student Research Grant, and for the Graduate Research Assistantship from the Department of Biology and Biotechnology Center. I wish to acknowledge the secretaries, faculty, and staff of the Biology Department for their assistance and support. I express sincere gratitude to my father, Mr. Eswaraiah Cheepala, mother, Mrs. Lakshmi Devi Cheepala, my brothers, Rajesh Cheepala, Mahesh Cheepala, and my sister-in-law Soni Cheepala, for their invaluable advice, encouragement, and support during my period of study and research.

Table of Contents

Page

IN VITRO REGENERATION AND GENETIC TRANSFORMATION OF *SESBANIA DRUMMONDII*: MEDICINALLY AND ENVIRONMENTALLY IMPORTANT PLANT

Satish babu Cheepala July 2003 Pages 55

Directed by: Shivendra V. Sahi, Sigrid Jacboshagen, and Byron Sleugh

Department of Biology, Department of Agriculture Western Kentucky University

This study describes rapid propagation of *Sesbania drummondii* using nodal explants isolated from seedlings and young plants. The nodal segments proliferated into multiple shoots on Murashige and Skoog (MS) medium supplemented with 22.2 μM benzyladenine. Murashige and Skoog medium containing 2.2 and 4.5 μM thidiazuron induced 5 - 6 healthier shoots per axillary node from 3-month-old plants. Nodal segments cultured on MS medium containing combinations of benzyladenine (8.8 and 11.1 μM) and either indole-3-butyric acid (0.24 - 2.46 μM) or indole-3-acetic acid (0.28 - 2.85 μM) produced fewer shoots. Callus when subcultured on 2.2 μM thidiazuron containing medium resulted in its mass proliferation of calli having numerous embryoid-like structures. Indole-3-butyric acid (0.24 - 2.46 μM) was found suitable for root induction. *In vitro* regenerated plants were acclimatized in greenhouse conditions. This report is a first report on studies of *in vitro* regeneration of *S. drummondii*.

Agrobacterium tumefaciens, bearing the plasmid pCAMBIA 1305.1, was used to develop a genetic transformation system for *S. drummondii*. This plasmid contains a *GUS* (β-glucuronidase) reporter gene and a gene conferring resistance to the antibiotic hygromycin. The genes are placed under the control of the 35S CaMV (cauliflower mosaic virus) promoter and a NOS terminator. A plant catalase intron is inserted into the *GUS* reporter gene.

The presence of *GUS* enzymatic activity, as a result of *Agrobacterium*-mediated transformation, was indicated by a GUS histochemical assay: the appearance of blue color in transformed tissue in the presence of substrate X-Gluc (5-bromo-4-chloro-3-indoly glucuronide). Polymerase Chain Reaction (PCR) was used to demonstrate the presence of the *GUS* reporter gene in the plant genome. Genomic DNA extracted from the transformed tissue was used to amplify the *GUS* gene by gene- specific primers. The PCR products were visualized by agarose gel-electrophoresis.

Chapter I

Introduction

Tissue culture of Sesbania drummondii

The use of tissue culture began early in the twentieth century when plant physiologists began studying the development of plant organs under defined conditions (Haberlandt 1902). After the discovery of auxins and cytokinins, tissue culture rapidly expanded as a technique in research. Since then tissue culture technology has been widely used for culturing various plant species. The technique is also called micropropagation.

Micropropagation has numerous advantages. For example, large numbers of pathogen-free plants can be produced in a short period of time. It also requires less starting material, and plants can be produced regardless of the season of the year (Kovac 1995). Rare and endangered plant species can be conserved and protected with the help of micropropagation (Mikulik 1999). Tissue culture is a prerequisite for developing transgenic plants with novel traits.

Sesbania drummondii (Rydb) Cory is a perennial legume shrub, commonly known as rattlebox drummond. It is distributed in seasonally wet places of southern coastal plains of the United States of America, from Florida to Texas. Seed extracts of this plant are significantly active against lymphocytic leukemia P-388 *in vivo.* Anti leukemic properties are attributed to the presence of medicinally important alkaloids: sesbanimide and sesbanine (Powell *et al.* 1976). These compounds, along with lignan Justicidin B, which was isolated later, have also been shown to have antineoplastic properties (Powell and

Smith 1981). In addition to the antitumor properties, a potent immunosuppressive activity has been reported for sesbanimides (Faircloth *et al.* 1996).

In addition to its being a source for the various pharmaceutically valuable compounds, *Sesbania drummondii* is a hyperaccumulator of toxic heavy metals like lead (Sahi *et al.* 2002), copper, and zinc (Sahi *et al.* 1999). Studies have shown that heavy metals, particularly Pb, are sequestered in aerial parts of this plant and it can therefore be an agent for phytoremediation of heavy metal contaminated sites. However an *in vitro* regeneration system has not been reported for this plant. Because of its importance, an alternative means to propagate this plant is essential.

Cell or tissue cultures of a number of plants have proven to be potential sources of secondary metabolites (alkaloids, glycosides, flavonoids, terpenoids) of pharmaceutical importance. Wang and Zong (2002) reported improved taxol production in suspension culture of *Taxus* species. Callus and cell suspension cultures are employed in the production of diterpenic acids from *Montanoa tomentosa* (Villarreal *et al.* 2001). Osuna *et al.* (2002) reported the production of sedative galphimine B by cell suspension culture of *Galphimia glauca*. Production of plumbagin from cell cultures of *Plumbago rosea* L. was also reported by Komaraiah *et al.* (2001). Therefore, it will be useful to study the tissue culture of *S. drummondii* for enhanced production of sesbanimides and sesbanine, the important secondary metabolites produced by this plant.

Sahi *et al.* (2002) have shown that lead taken up by *S. drummondii* is sequestered in the cell wall and plasma membrane of this plant. Gardea-Torresdey *et al.* (1990) reported that carboxyl groups in cell walls of dead algal biomass are partially responsible for metal binding. Therefore, increasing the cell wall content of a plant through genetic

transformation may enhance the capacity to hyperaccumulate lead. Moreover, hyperaccumulators can also be achieved by selecting cell-lines of *Sesbania* with increased cell wall content. Tissue cultures of *S. drummondii* can generate a large number of identical clonal plants endowed with unique phytoremediation potential and enhanced production of sesbanimides.

Considerable attention has been paid to the development of tissue culture techniques in order to improve legume crops. Most of this work has been done with economically important herbaceous legumes such as pea (Kartha *et al.* 1974) and alfalfa (Saunders and Bingham 1975). There is a growing interest in applying similar techniques to woody legume species in order to improve multipurpose legume crops. Several genera of woody legumes including *Acacia* (Jones *et al.* 1990) have now been successfully cultured *in vitro* for micropropagation or plant regeneration purposes.

The genus *Sesbania* contains approximately 70 species that are widespread throughout the tropics and subtropics. All *Sesbania* species are nodulated by nitrogen fixing *rhizobia*, allowing them to grow rapidly on nitrogen poor soils. Many species can also survive a wide range of soil types such as arid, waterlogged, or alkaline soils (Evans and Rotar 1987). They are potentially useful for providing ground cover to improve soil fertility and control erosion. Certain species are also used in plantations as sources of pulp for paper production, fiber, fodder, gums, and medicines, and the trees may also be used as ornamentals (Evans and Rotar 1987). Several species are woody annuals or short-lived perennials, representing an intermediate stage between herbaceous annuals and long-lived woody perennial trees.

Reports of *in vitro* culture of the genus *Sesbania* have included tissue culture and micropropagation of several species. In *S. rostrata* conditions for callus induction and *in vitro* morphogenesis have been optimized using cotyledon, hypocotyl and immature embryo explants (Vlachova *et al.* 1987). Direct shoot bud formation was observed in 10 – 20 % of the hypocotyl explants cultured on MS medium containing 1 mg/L benzyladenine (BA) concentration. Direct shoot formation did not occur from the cotyledons explants when grown on the same medium. However, seedling explants preconditioned on medium containing high concentration of BA (2 mg/L) were capable of direct shoot induction at low frequency when cultured at the concentration of 1 mg/L BA. 1-Napthaleneacetic acid (NAA) at 0.1 mg/L was found to stimulate root development of the regenerated plantlets.

In vitro regeneration using cotyledon explants was achieved by Detrez *et al.* (1994) in *S. grandiflora*. Murashige and Skoog media containing BA and NAA was used to develop *in vitro* micro- propagation of *S. grandiflora*. This research showed that dark grown seedlings are optimal for an efficient and reproducible bud induction. Approximately 96 % of the explants yielded more than 30 buds per explant in a week. Histological studies showed that bud induction in *S. grandiflora* occurs by the direct organogenetic pathway.

In *S. cannabina, S. bispinosa, S. formosa and S. sesban*, Yan-Xiu *et al.* (1993) have shown callus mediated shoot regeneration from hypocotyl and cotyledon explants using various concentrations of BA, in combination with indole-3-buytric acid (IBA), NAA and 2,4-dichlorophenoxyacetic acid (2,4-D). Medium containing 2,4-D or NAA produced the fastest growing callus, and shoots were readily regenerated from hypocotyls and

cotyledons explants. Frequent shoot regeneration was observed with IBA (0.25 - 4.92 μM) in combination with BA (4.44 - 8.8 μM), but shoot regeneration was not observed in the presence of 2,4-D. All of the explants developed callus. Shoots that differentiated were excised and cultured on MS medium containing IBA (2.46, 4.92 and 9.84 μM) for root induction, which appeared after 3 - 8 days of culture.

Kapoor and Gupta (1986) achieved rapid *in vitro* differentiation of *S. bispinosa* using hypocotyl and cotyledon explants. Multiple shoots differentiated from hypocotyl explants when cultured on Gamborg's basal medium containing BA (0.1 - 10 μM). Cotyledons differentiated multiple shoots when cultured on medium containing BA (1 -10 μM). Hypocotyls produced 10 - 12 shoots per explant at low concentrations of BA, whereas frequencies of shoots were low at higher concentrations of BA. On the other hand the shoots did not differentiate from cotyledons when cultured on lower concentrations of BA (< 1 μM).

Yan-Xiu *et al.* (1995) reported plant regeneration from protoplasts isolated form cotyledons of *S. bispinosa*. When protoplasts were cultured on MS medium supplemented with 1 mg/L 2,4-D, 2 mg/L BA, 1 mg/L glutamine and 0.5 M mannitol, 84 % of the protoplasts divided and formed callus. These calli were transferred to MS medium containing 1 mg/L IBA and 1 mg/L BA differentiated into shoots. These shoots developed into complete plantlets when excised and cultured on MS medium supplemented with 0.5 mg/L IBA.

Recently, Shahana and Gupta (2002) reported somatic embryogenesis in *S. Sesban* from the cotyledons. They showed that 23 % of the cultures developed on an average of

9.3 embryos per cotyledonary explant when cultured on LS medium supplemented with 0.1 mg/L NAA.

From the above studies on the tissue cultures of various *Sesbania* species, we can conclude that the different concentration of BA alone or in combination with IAA or IBA or NAA favored shoot induction. However, the morphogenetic response of the various *Sesbania* species is very unique among species and also the response is species specific.

S. drummondii has escaped the attention of researchers, despite the fact that plant regeneration from cultured *Sesbania* tissues provides the basis for possible cell culture and genetic manipulation as well as a potential plant source for phytoremediation. There are no published reports on any kind of plant cell and tissue culture work on this particular species. Plant cell culture of *Sesbania* can be a potential source of phytopharmaceuticals. Furthermore, *in vitro* regenerated plants can be a source for commercial phytoremediation applications. Therefore, there is a need to develop a method for rapid regeneration of plantlets. *In vitro* regeneration systems are also a prerequisite for development of genetic manipulation of *Sesbania* to improve its application.

Genetic Transformation

The study of tumor formation in plants has made tremendous and interesting progress since Smith and Townsend (1907) showed that *Agrobacterium tumefaciens* induces crown gall tumors in plants. Landmark discoveries and pertinent investigations were made in the 1970's and 1980's. It was found that tumor formation involved transfer of oncogenic segments of DNA (Chilton *et al.* 1977), and that this response occurred in the presence of host-released chemical signals (Johnson and Das, 1998). The bacterium

inhabits soil, and its attachment to plant cell takes place in response to phenolic compounds released at plant wound sites (Winans *et al.* 1994). Tumor formation occurs by transfer and integration of the T-DNA region present in the tumor-inducing plasmid (Ti-plasmid) of *Agrobacterium* into the plant genome. The T-DNA fragment contains phytohormone biosynthesis genes responsible for gall formation in plants. The other type of genes in the T-DNA regions encodes enzymes for the synthesis of opines. *Agrobacterium* infection of plants results in tumor formation and the release of opines (Graves and Goldman, 1986). The opine metabolism genes in the Ti plasmid synthesize opines like nopaline, octopine, and succinopine which are used as energy sources by *Agrobacterium* (Hooykaas *et al.* 1984).

The Ti plasmid of *Agrobacterium* is flanked on either side by 25 base pairs direct repeats. The process of T-DNA transfer is a cooperative action of the different proteins encoded by the virulence (*vir*) genes and other proteins in the bacterial chromosome. The virulence region located on Ti-plasmid is about 30 kb and comprises six operons essential for T-DNA transfer, called *virA, B, C, D, G* and *E* (Binns and Thomashow, 1988). The T-DNA transfer takes place in an organized and stepwise manner. The primary process is the attachment of the bacteria to the plant cell where the bacteria colonize and anchor onto the host cell.

The *virA* and *virG* are responsible for activating the transcription of other virulence genes (Hansen *et al.* 1994). VirA is a transmembrane dimeric sensor protein that detects signal molecules. The signals include plant phenolics like acetosyringone (AS) released from wounded plant tissue (Stachel *et al.* 1985).

Chang and Winans (1992) found that VirA protein structurally has three domains -- the periplasmic or input domain and two transmembrane domains, TM1 and TM2, which act as transmitters of signals and also sensors for receiving signals. The periplasmic domain is considered essential for detection of monosaccharides. TM2 plays a critical role in the activation of VirA by autophosphorylation at a conserved His - 474 residue in response to signaling molecules from injured/wounded plants. Once the VirA is activated, it transfers its phosphate to a conserved aspartate residue on the cytoplasmic DNA binding protein VirG. VirG acts as a transcription factor, regulating the expression of virulence genes when it is phosphorylated by VirA (Jin *et al.* 1990).

The right border of the T-DNA region is imperative for T-DNA transfer (Hille *et al.* 1983). Synthesis of T-DNA is initiated at the right border and proceeds in the 5' to 3' direction. The enhancer present next to the right border is specifically recognized by VirC1. This enhancer is shown to increase the virulence of *Agrobacterium* strains (Toro *et al.* 1989).

VirD1 and VirD2 proteins are involved in recognizing and nicking the bottom strand of T-DNA. After the endonucleolytic cleavage, VirD2 remains covalently attached to the 5' end of the single stranded T-DNA and distinguishes the 5' end as the leading end of the transfer complex (Durrenberger *et al.* 1989). This complex is now ready for transfer to the plant cell and must be translocated to the plant nucleus for integration into the plant genome. The VirB protein forms a channel between the bacterial and plant cell for T-DNA transfer. Once the naked single-stranded T-DNA complex is inside the plant cell it is coated with VirE2 protein, which protects it from degradation by plant nucleases and also assists in integration (Das 1988). VirE2 and VirD2 proteins contain two plant

nuclear localization signals (NLS) responsible for directing the T-DNA complex into the plant nucleus (Zupan and Zambryski, 1995).

In order to develop transgenic plants, the tumor-causing genes on the T-DNA are disarmed (Barton *et al.* 1983; Fraley *et al.* 1985). Next, novel genes of interest, along with selectable marker genes like antibiotic resistance genes, are inserted between the border sequences of the T-DNA region (Bevan *et al.* 1983). Efforts to generate modified plants using *Agrobacterium*-mediated transformation have been successful in most dicotyledon species. Recent reports indicate development of pest resistant transgenic cotton plants via expression of the *B. subtilis* Protox gene. An ubiquitin promoter was attached to this gene and targeted to the cytoplasm and plastid using *Agrobacterium*-mediated gene transformation (Lee *et al.* 2000). *Agrobacterium*-mediated transformation is a most widely used technique for genetic transformation because of its simplicity and efficiency in providing stable integration of transferred DNA into plant genomes (Koroch *et al.* 2002).

Susceptibility to *A. tumefaciens* infection was examined by gall formation near the infected site in *S. rostrata* by Vlachova *et al.* (1987) using various wild type strains of *A. tumefaciens* (C58 and B6S3) and *A. rhizogenes* (15834). An extensive systemic infection of *S. rostrata* by the *Agrobacterium* strains was observed, presumably occurring via spread of the bacteria in the vascular bundles. This study has opened the way for transformation experiments using *Agrobacterium* strains carrying binary vector and antibiotic resistant genes.

Chunhai *et al.* (1991) reported genetic transformation of *Sesbania* species (*S. bispinosa, S. cannabina, S. formosa* and *S. sesban*) with *A. tumefaciens* (B6S3 and LBA4404) strains. In this study they found that none of the uninfected explants could survive or produce callus on the cefatoxine containing MS (Murashige and Skoog 1962) medium. In contrast, *Agrobacterium*-infected explants was able to produce callus on hormone independent medium. It clearly showed the susceptibility of the *Sesbania* explants to *Agrobacterium* infection. Analysis of opines in callus tissue from the infected explants indicated the presence of octopine, a compound found only in transformed tissue. The study demonstrated the stable transformation of the various explants, showing that *S. formosa* hypocotyl explants were more readily transformed than cotyledons. Among the four species, *S. formosa* gave the highest frequency of transformation.

Studies on genetic transformation of *Sesbania* species are not exhaustive. Most of the earlier work involved susceptibility of the plant and the ability of the *Agrobacterium* strains to infect. We filled the gap by initiating novel studies on the development of genetic transformation of *S. drummondii* by expressing a *GUS* gene in callus and embryoid tissue. *S. drummondii* represents a model system for selection and genetic improvement of this taxon, and should be considered a potential candidate for genetic improvement using the techniques of plant biotechnology.

The present study was focused on *in vitro* morphogenesis and genetic transformation. The thesis is divided into two sections: the first section chapter II deals with *in vitro* regeneration of the *S. drummondii* using various explants. The second section chapter III deals with the genetic transformation, genetic transformation was achieved using *Agrobacterium tumefaciens*-mediated transformation in the nodal explants.

References

Barton, K.A., Binns, A.N., Matzke, A.J., Chilton, M.D.: Regeneration of intact tobacco plants containing full length copies of genetically engineered T-DNA, and transmission of T-DNA of R1 progeny. - Cell **32**: 1033-43, 1983

Bevan, M.W., Flavell, R.B., Chilton, M.D.: A chimeric antibiotic resistance gene as a selectable marker for plant cell transformation. - Nature **304**: 184-187, 1983

Binns, A.N., Thomashow, M.F.: Cell biology of *Agrobacterium* infection and transformation of plants. Annu. Rev. Microbiol. **42**: 575-606, 1988.

Chang, C.H., Winans, S.C.: Functional roles assigned to the periplasmic, linker and receiver domains of the *Agrobacterium tumefaciens* VirA protein. J. Bacteriol **174**: 7033-7039, 1992

Chilton, M.D., Drummond, M.H., Merlo, D.J., Sciaky, D., Monotoya, A.L., Gordon M.P., Nester E.W.: Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorigenesis. – Cell **11:** 263-271, 1977.

Chunhai, D., Dunyi, Y., Harris, P.J.C.: Genetic transformation of *Sesbania* species with *Agrobacterium tumefaciens*. Nitrogen fixing tree Res. Reports **9**: 134-136, 1991.

Detrez, C., Ndiaye, S., Dreyfus, B.: *In vitro* regeneration of the tropical multipurpose leguminous tree *Sesbania grandiflora* from cotyledon explants. - Plant Cell Rep. **14**: 87-93, 1994.

Das, A.: *Agrobacterium tumefaciens virE* operon encodes a single-stranded DNA-binding protein. - Proc. Natl. Acad. Sci. USA, **85**: 2209-2213, 1988.

Durrenberger, F., Crameri, A., Hohn, B., Koukolikova-Nicola, Z.: Covalently bound Vir D2 protein of *Agrobacterium tumefaciens* protects the T-DNA from exonucleolytic degradation. Proc. Natl. Acad. Sci. USA, **86**: 9154-9158, 1989.

Evans, D.O., Rotar, P.P. *Sesbania* in agriculture. Westview tropical agriculture series No. 8 (pp 1-4). West view Press, Boulder and London.

Faircloth, G.T., Romero, F., Canedo, L.M., Acebal, C.: Immunosuppressive pharmaceutical compositions new biological activity from a marine *Agrobacterium* sp. - United States Patent No. **5**, 556,777, 1996.

Fraley, R.T., Eichholtz, D.A., Flick, J.S., Fink, C.L., Hoffmann, N.L. Sanders P.R.: The SEV system: a new disarmed Ti plasmid vector system for plant transformation. – Biotechnol. **3**: 629-635, 1985.

Graves, A.C.F., Goldman, S.L.: Transformation of *Zea mays* seedling with *Agrobacterium tumefaciens*. Plant Mol. Biol. **7**: 43-50, 1986.

Gardea-Torresdey, J.L., Beeker-Hapaak, M.K., Hosea, J.M., Darnall, D.W.: Effect of chemical modification of algal carboxyl groups on metal binding. Environ. Sci. Technol. **24**: 1372, 1990.

Haberlandt, G.: Kulturversuche mit isolierten Pflanzenzellen. Sitzungsber. Mat. Nat. Kl. Kais. Akad. Wiss. Wien. **111**: 69-92, 1902.

Hansen, G., Das, A., Chilton, M.D.: Constitutive expression of virulence genes improves the efficiency of plant transformation by *Agrobacterium*. Proc. Natl. Acad. Sci. USA **91**: 7603-7, 1994.

Hille, J., Wullems, G., Schilperoot, R.A.: Non-oncogenic T-region mutants of *Agrobacterium tumefaciens* do transfer T-DNA into plants. - Plant Mol. Biol. **2**: 155-163, 1983.

Hooykaas, G.M.S., Slogteren, V., Hookyaas, P.J.J., Schilperoot, R.A.: Expression of Ti plasmid genes in monocotyledons plants infected with *Agrobacterium tumefaciens*. - Biotechnology **24**: 382-383, 1984.

Jin, S., Prusti, R.K., Roitisch, T., Ankenbauer, R.G., Nester, E.W.: The VirG protein of *Agrobacterium tumefaciens* is phosphorylated by the autophosphorylated VirA protein and this is essential for its biological activity. - J. Bacteriol. **172**: 4945-4950, 1990.

Johnson, T.M., Das, A.: Organization and regulation of expression of the *Agrobacterium* virulence genes, 265-279. In Spaink HP, Kondorosi A, and Hooykaas PJJ (ed.), The Rhizobiaceae: Molecular biology of model plant-associated bacteria. Kluwer Academia Publishers, Dordrecht, The Netherlands 1998.

Jones, T.C., Batchelor, C.A., Harris, P.J.C. *In vitro* culture and propagation of *Aacia* species (*A. bivenosa, A. salicina, A. saligna and A. sclerosperma*). – Intl. Tree Crops. J. **6**: 183-192, 1990.

Kapoor, S., Gupta, S.C.: Rapid *in vitro* differentiation of *Sesbania bispinosa* plants – a leguminous shrub. - Plant Cell Tiss. Org. Cult. **7**: 263-268; 1986.

Kartha, K.K., Gamborg, O.L., Constable, F.: Regeneration of Pea (*Pisum sativum* L.) plants from apical meristems. - Z. Pflanzenphysiol. **72**: 177-176, 1974.

Komaraiah, P., Kishor, P.B.K., Ramakrishna, S.V.: Production of plumbagin from cell cultures of *Plumbago rosea* L.- Biotech. Let. **23**: 1269-1272, 2001.

Koroch, A., Kapteyn, J., Juliani, H.R., and Simon J.E.: *In Vitro* regeneration and *Agrobacterium* transformation of *Echinacea purpurea* leaf explants. Trends in new crops and new uses, 2002. J. Janick and A. Whipkey (eds.). ASHS Press, Alexandria, VA.

Kovac, J.: Micropropagation of *Dianthus arenarius* subsp. *Bohemicus* an endangered endemic species from Czech Republic.- Bot. Gard. Micropropagation news **8**: 106-108, 1995.

Lee, H.J., Lee, S.B., Chung, J.S., Han, S.U., Han, O., Guh, J.O., Jeon, J.S., An, G., Back, K.: Transgenic rice plants expressing a *Bacillus subtilis* protoporphyrinogen oxidase gene are resistant to diphenyl ether herbicide oxyflurofen. - Plant Cell Physiol. **41**: 743-749, 2000.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant. **15**: 473-497, 1962.

Mikulik, J.: Propagation of endangered plant species by tissue cultures. - Biologica **37**:27-33, 1999.

Osuna, L., Miranda, P.R., Villareal, M.L.: *In Vitro* production of sedative galphimine B by cell suspension cultures of *Galphimina glauca*. – Biotech. Let. **24**: 257-261, 2002.

Powell, R.G., Smith, C.R.: An investigation of the anti-tumor activity of *Sesbania drummondii*. - J. Nat. Prod. **44**: 86-90, 1981.

Powell, R.G., Smith, C.R., Madrigal, R.V.: Antitumor activity of *Sesbania vesicaria*, *S. punicea* and *S. drummondii* seed extracts. Planta Medica. **30**: 1-8, 1976.

Saunders, J.W., Bingham, E.T.: Growth regulators effects on bud initiation in callus cultures of *Medicago sativa*. – Amer. J. Bot. **62**: 850-855, 1975.

Sahi, S.V., Bryant, N.L., Sharma, N.C., Singh, S.R.: Characterization of a lead hyperaccumulator shrub, *Sesbania drummondii*. - Environ. Sci. Technol. **36**: 4676-80, 2002

Sahi, S.V., Vanosia, F., Bryant, N., Andersland, J.: Accumulation of heavy metals by *Sesbania* species. Proc. 5th International Conference on the Biogeochemistry of Trace Elements, July 11-15, 1999, Vienna, Austria. 582-583, 1999.

Shahana, S., Gupta, S.: Somatic embryogenesis in *Sesbania sesban* var. *bicolor*: A multipurpose fabaceous woody species - Plant Cell Tiss. Org. Cult. **69**: 289-292; 2002.

Smith, E.F., Townsend, C.O.: A plant tumor of bacterial origin.- Science **25**:671-673, 1907.

Stachel, S.E., Messens, E., Van Montagu, M., Zambryski, P.: Identification of signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens*. -Nature **318**: 624-629, 1985.

Toro, N., Datta, A., Carmi, O.A., Young, C., Prusti, R.K., Nester, E.W.: The *Agrobacterium tumefaciens* VirC1 gene products binds to overdrive, a T-DNA transfer enhancer. - J. Bacteriol. **171**: 6845-6849, 1989.

Vlachova, M., Merz, B.A., Schell, J., De Brujin, F.J.: The tropical legume *Sesbania rostrata*: tissue culture, plant regeneration and infection with *Agrobacterium tumefaciens* and *rhizogens* strains. Plant Sci. **50**: 213-223, 1987.

Villarreal, M.L., Rojas, G., Quintero, R., Miranda, E., Enriquez, L.I., Reynolds, W. : *In Vitro* culture of *Monatano tomentosa* for the production of diterpenic acids. Biotech. Let. **23** (16): 1279-1284, 2001.

Wang, Z.Y., Zong, J.J.: Repeated elicitation enhances taxane production in suspension cultures of *Taxus chinensis* in bioreactors. Biotech. Let. **24**: 445-448, 2002.

Winans, S.C., Mantis. N.J., Chen, C.Y., Chang, C.H., and Han, D.C.: Host recognition by VirA, VirG two component regulatory proteins of *Agrobacterium tumefaciens*. - Res. Microbiol. **145**: 461-473, 1994.

Yan-Xiu, Z., Dun-Yi., Harris, P.J.C.: Plant regeneration from callus and explants of *Sesbania* spp. - Plant Cell Tiss. Org. Cult. **34**: 253-260, 1993.

Zupan, J.R., Zambryski, P.C.: Transfer of T-DNA from *Agrobacterium* to the plant cell. - Plant Physiol. **107**: 1041-1047, 1995.

Chapter II

Rapid *in vitro* regeneration of *Sesbania drummondii*

Abstract

Sesbania drummondii (Rydb) Cory (Rattlebox) produces medicinally important compounds that show great potential as anti-leukemic and immunosuppressant agents. Rapid *in vitro* propagation of *Sesbania drummondii* was achieved using various nodal explants (cotyledonary and axillary branch node). Nodes were cultured on MS medium containing various concentrations and combinations of cytokinin and auxins for differentiation. Maximum number of shoots was obtained from cotyledonary node on MS medium supplemented with BA in a span of four weeks. These explants also produced shoots and profuse callus on the MS medium containing 0.5 mg/l thiadiazuron.

Introduction

Sesbania drummondii (Rydb) Cory (Fabaceae) is a perennial shrub, commonly known as rattlebox drummond. It is distributed in seasonally wet places of southern coastal plains of the United States of America, from Florida to Texas.

The seeds of this plant have been proven to contain medicinally important alkaloids: sesbanimide and sesbanine (Powell and Smith. 1981). Sesbanimide acts as an antineoplastic agent in animals, while sesbanine causes remission in leukemic tumors in animals (Powell *et al.* 1976). Recently, *Sesbania drummondii* has been shown to have promise in removal of toxic metals from environment. It has been reported to phytoremediate significant amounts of lead (Pb), copper (Cu), and zinc (Zn) from contaminated aqueous solutions and soil (Sahi *et al.* 2002, Sahi *et al.* 1999).

Of a wide range of *Sesbania* species, *in vitro* regeneration protocols have been developed for a few species, notably the following: *S. rostrata* (Vlachova *et al.* 1987), *S. sesban* (Khattar and Mohan Ram 1982, Harris and Puddephat 1989, Yan- Xiu *et al.* 1993), *S. grandiflora* (Khattar and Mohan Ram 1983, Shanker and Mohan Ram 1990), *S. cannabina* (Xu *et al.* 1984), *S. bispinosa* (Kapoor and Gupta 1986, Sinha and Mallick 1991) and *S. rostrata* (Hanower *et al.* 1986). In these species, plant regeneration has been obtained by enhanced axillary branching as well as callus-mediated organogenesis from seedling explants and greenhouse-grown plant parts. There is no systematic cultivation of this plant, and no published reports on tissue cultures of *Sesbania drummondii* are available. Therefore, there is a need to develop a means for rapid regeneration of plantlets from *Sesbania* cultures. The present study focuses on *in vitro* morphogenesis and plant regeneration using various seedling explants such as cotyledonary node, axillary branch node, hypocotyl, epicotyl, cotyledon, and stem segments from greenhouse-grown *Sesbania* plants.

Materials and methods

Seed germination

Seeds of *Sesbania drummondii* were scarified using 85 % H_2SO_4 for 30 min followed by washing thoroughly under running tap water for 1-h. Seeds were sterilized with 0.2 % mercuric chloride ($HgCl_2$) and then transferred to magenta boxes containing water–agar (0.6 %), and incubated at 25 – 28 °C under 16-h photoperiod of 20 μmol m^{-2} s^{-1} irradiance provided by cool-white fluorescent tubes.

Explant preparation

Ten-day-old seedlings were harvested for use of different explants: CN (cotyledonary node) (1.5 – 2 cm segment of embryonic axis bearing nodes without cotyledons), AN (axillary branch node), hypocotyl, and epicotyl. Individual cotyledons were excised from sterilized seeds soaked for 48-h on water-agar medium. Stem segments (bearing nodes) excised from 3-month-old greenhouse-grown plants were also used. Explants were surface sterilized with 0.1 % $HgCl_2$ for five minutes and then rinsed three times with sterile deionized water.

Media and culture conditions

MS (Murashige and Skoog 1962) medium was supplemented with various concentrations of plant growth regulators: 4.4 – 22.2 μM benzyladenine (BA); 8.8 μM, 11.1 μM BA + 0.24 2.46 μM indole-3-butyric acid (IBA); 8.8 μM, 11.1 μM BA + 0.28 – 2.85 μM indole-3-acetic acid (IAA); 4.4 - 22.2 μM BA + 1.34 8.0 μM napthaleneacetic acid (NAA) and 2.27 – 4.54 μM thidiazuron (TDZ). The basal (MS)

medium also contained 30 g l^{-1} of sucrose and 8 g l^{-1} agar as gelling agent; the pH of the media was adjusted to 5.8 after adding plant growth regulators. After sterilization (121 °C, 20 min), the medium (15 ml each) was dispensed into culture tubes (150 x 25 mm), which were plugged with Kim caps (*Fisher Scientific Co.*, USA). Explants were transferred to culture tubes containing different concentrations of plant growth regulators. These cultures were incubated at 25 ± 2 °C under 16-h photoperiod of 50 µmol m^{-2} s^{-1} irradiance provided by cool-white fluorescent tubes for 35 days. Each experiment consisted of 12 explants (in different culture tubes) and was repeated twice. Callus cultures were maintained by regular subculturing at every 4 5 weeks on fresh medium.

Rooting and acclimatization

For rooting, 4 - 5 cm long regenerated shoots were transferred to MS medium alone and in presence of 0.24 – 2.46 μM IBA as well. After plantlets attained height of 8 - 10 cm, they were transferred to plastic cups containing autoclaved peat moss. They were irrigated with half-strength Hoagland's medium and covered with a plastic wrapper to prevent environmental shock as a result of humidity loss. Plants were maintained at 25 ± 2 °C and 60 – 70 % relative humidity under bright daylight. Plants were frequently watered and gradually exposed to the natural environment.

Statistical analysis

Statistical analysis of data was carried out by using SYSTAT (version 9.0 for Windows, 1999, *Systat Software Inc.*, Richmond, California). Observations were recorded for the frequency (percent of cultures responding to shoot proliferation, callusing and root development) and the numbers of shoots per explant and shoot length. The analysis of variance (ANOVA) appropriate for the design was carried out to detect

the significance of differences ($P < 0.05$) among the treatment means, and a Tukey HSD post hoc test was performed to compare among the groups for significant differences

Results and Discussion

Axillary bud proliferation

Axillary branch node (AN) and cotyledonary node (CN) segments of 10-day-old seedlings proliferated into multiple shoots on MS medium supplemented with 4.4 - 22.2 μM BA. With increase in concentration of BA, the number of shoots per explant increased. The maximum number of shoots per CN explant was achieved using 22.2 μM of BA (Table 1 and Fig. 1A). Of the two explants, CN produced the higher number of shoots per explant (< 8), and longer shoots were developed within four weeks. However, the percent cultures showing regeneration were comparable in both explants (Table 1). Increasing BA concentration above 13.3 μM, however increased axillary bud proliferation in CN but resulted in reduced growth of shoots.

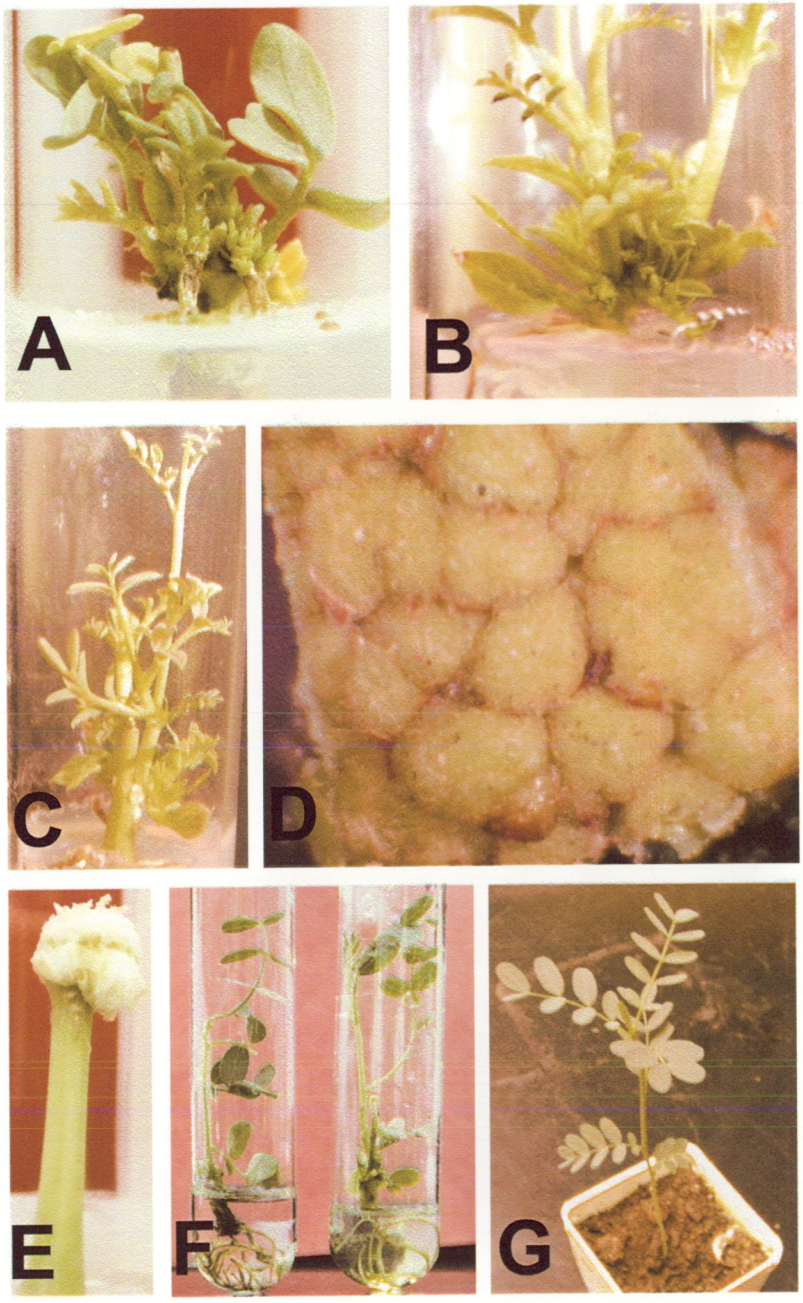
Axillary branch nodes excised from 3-month-old plants proliferated into multiple shoots on MS medium containing 2.27 and 4.54 μM TDZ. TDZ induced 5 - 6 shoots per explant (Fig. 1B) that were healthier than those regenerated on BA. But the frequency of the regenerants varied from 50 – 60 % at both concentrations of TDZ. The basal part of each stem proliferated into large callus mass, which could not differentiate further. TDZ-induced multiple shoot regeneration by bud break has been reported in a number of woody species (Murthy *et al.* 1998). TDZ has also been shown to promote differentiation of organized centers of growth in cultured tissues at much lower concentrations, and shoot regeneration occurs with efficiency comparable to or greater than that of other cytokinins (Malik and Saxena 1992). In this study, TDZ at 2.27 μM or 4.54 μM was

Table 1. Shoot proliferation from *S. drummondii* cotyledonary node (CN) and axillary branch node (AN) explants (excised from seedlings) and stem node (excised from 3-month-old plants) following 5 weeks of culture on different concentrations of BA and TDZ. Each mean is based on three sets of replicates, each of which consisted of 12 cultures tubes.

BA [μM]	Shoot length [cm]		Number of shoots [explant]		Regeneration [%]	
	CN	AN	CN	AN	CN	AN
4.4	2.5^{a*}	1.5^{a}	2.63^{a}	2.18^{a}	90	90
8.8	2.5^{a}	1.0^{b}	5.0^{b}	3.58^{b}	90	90
11.1	2.0^{ca}	1.0^{b}	4.75^{b}	3.83^{b}	100	100
13.3	2.0^{ca}	0.75^{c}	6.5^{c}	4.08^{c}	90	90
18.5	1.5^{b}	0.5^{d}	7.08^{d}	2.66^{b}	80	80
22.2	1.5^{b}	0.5^{d}	7.75^{d}	1.5^{a}	80	80
Stem node TDZ μM						
2.27	-	2.7^{a}	-	6.2^{a}	-	60
4.54	-	2.5^{a}	-	5.0^{b}	-	50

*Means having different letters in superscript are significantly different from each other ($P < 0.05$) within the same column according to *ANOVA* and Tukey HSD multiple comparisons post hoc test.

Fig 1. Morphogentic response of *Sesbania drummondii* explants. A Induction of multiple shoots from cotyledonary node on MS medium containing 22.2 μM BA. B Stem node (from 3-month-old plant) showing enhanced branching on MS medium containing 2.27 μM TDZ. C - Proliferation of elongated shoots from axillary node on MS medium supplemented with 11.1 μM BA and 2.46 μM IBA. D Cotyledon explant subcultured on MS medium with 2.27 μM TDZ showing proliferation of callus with embryoid-like structures. E Hypocotyl explant cultured on 4.4 μM BA and 2.69 μM NAA showing callus on the apical part. F - Elongated shoots with roots (complete plantlets). G - Acclimatized *Sesbania drummondii* plant growing on peat moss.


A
B
C
D
E
F
G

found to be comparable with BA-induced regeneration at higher concentrations (13.3 – 22.2 μM) (Table 1).

When axillary and cotyledonary node explants were cultured on MS medium containing combinations of 8.8 μM and 11.1 μM BA, and 0.24 μM to 2.46 μM IBA, cotyledonary nodes showed higher frequency of shoot regeneration than axillary nodes (Table 2). Cotyledonary node also produced larger shoots than the axillary node except at the concentration of 8.8 μM BA and 2.46 μM IBA. The best combination for cotyledonary node proliferation was 11.1 μM BA and 2.46 μM IBA (Table 2). The frequency of shoot regeneration was 30 - 90% in cotyledonary node explants while 30 - 50% in axillary branch node. Out of many regenerated shoots, one or two shoots were dominantly elongated on these combinations of BA and IBA (Fig. 1C).

MS medium supplemented with different concentrations of BA (8.8 μM and 11.1 μM) and IAA (0.28 μM – 2.85 μM) induced regeneration of shoots (50 - 90%) from both CN and AN explants (Table 3). Maximum number of shoots produced per explant was > 4 in CN, whereas number of shoots per AN explant was $\leq$ 3. The average shoot length was between 1.0 - 4.0 cm for CN and 0.75 - 2.5 cm for AN (Table 3). BA (11.1 μM) in combination with IAA (2.85 μM) induced rooting at the base of regenerated shoots in about 10 % of cotyledonary node explants. This event in *Sesbania drummondii* is comparable to the results in *Sesbania aculeate* (Bansal and Pandey 1993). Differentiation

Table 2. Number, length and percentage of shoots from cotyledonary node (CN), axillary branch node (AN) explants of *S. drummondii* following 5 weeks of culture on different concentrations and combinations of BA and IBA. Each mean is based on three sets of replicates, each of which consisted of 12 culture tubes.

BA [μM]	IBA [μM]	Shoot length [cm]		Number of shoots [explant]		Regeneration [%]	
		CN	AN	CN	AN	CN	AN
8.8	0.24	1.0^{a*}	1.0^{a}	3.16^{a}	1.66^{a}	75	50
8.8	0.49	3.5^{b}	2.0^{b}	3.0^{b}	1.25^{a}	70	50
8.8	2.46	1.0^{a}	1.5^{a}	1.4^{c}	0.8^{b}	50	40
11.1	0.24	1.0^{a}	1.0^{a}	1.66^{c}	1.0^{b}	30	30
11.1	0.49	1.5^{b}	1.0^{a}	3.5^{a}	2.0^{c}	75	50
11.1	2.46	7.5^{c}	2.5^{c}	4.77^{d}	1.18^{a}	90	40

*Means having different letters in superscript are significantly different from each other ($P < 0.05$) within the same column according to *ANOVA* and Tukey HSD multiple comparisons post hoc test.

Table 3. Number, length and percentage of shoots from cotyledonary node (CN), axillary branch node (AN) explants of *S. drummondii* following 5 weeks of culture on different concentrations of BA and IAA. Each mean is based on three sets of replicates, each of which consisted 12 cultures tubes.

BA [μM]	IAA [μM]	Shoot length [cm]		Number of shoots [explant]		Regeneration [%]	
		CN	AN	CN	AN	CN	AN
8.8	0.28	1.0^{a*}	0.75^{a}	2.28^{a}	0.8^{a}	75	50
8.8	0.57	2.5^{b}	2.0^{b}	1.125^{b}	0.8^{a}	60	50
8.8	2.85	2.5^{b}	NA	3.6^{c}	NA	50	NA
11.1	0.28	1.0^{a}	1.0^{a}	2.33^{a}	0.6^{a}	90	90
11.1	0.57	3.5^{c}	2.5^{c}	3.5^{b}	1.5^{b}	75	50
11.1	2.85	4.0^{d}	2.5^{c}	4.27^{c}	3.0^{c}	90	90

*Means having different letters in superscript are significantly different from each other ($P < 0.05$) within the same column according to *ANOVA* and Tukey HSD multiple comparisons post hoc test.

of shoot and root simultaneously from primary explants in presence of BA and IBA hints towards some sub-optimal function of somatic embryogenic pathway. Some of the epidermal cells surrounding axillary bud might be induced to form embryoids, leading to regeneration of complete plants. Similar observations were also made in *Glycine wightii* (Pandey and Bansal 1992).

Multiplication of shoots

Shoots regenerated from axillary branch nodes (from 3-month-old plants) on TDZ medium were excised and elongated individually on MS medium supplemented with 1.14 μM IAA. Elongated shoots having a minimum of two nodes each were harvested and cut into two pieces, each containing a nodal segment. Subsequently, nodal segments were transferred to MS medium containing 2.27 μM TDZ for stage II multiplication. In four weeks, each axillary bud proliferated about 10 shoots. Thus 100-fold multiplication could be achieved from each primary explant in a span of 9 -10 weeks.

Establishment of callus culture

Two-day-old cotyledons excised from seeds were cultured on MS medium containing different concentration of BA (2.2 13.3 μM). They produced calli on cotyledonary margins more or less on all concentrations of BA. Subculturing of these callus pieces on MS medium containing 2.27 μM TDZ resulted in a large callus mass containing a number of shiny, globular structures, which resembled like embryoids (Fig. 1D). These callus cultures were maintained for three months by regular subculturing. However, various attempts to stimulate embryogenesis or regeneration could not be achieved. BA induced

multiple shoot regeneration directly from cotyledon has been reported in *S. bispinosa* (Kapoor and Gupta 1986). In *S. grandiflora* rapid bud proliferation from cotyledonary explants has been shown as a means of mass propagation (Detrez *et al.* 1994). It has also been shown in *S. grandiflora* that lighting conditions and age of donor seedlings critically affect shoot organogenesis from cotyledon. Inability of *S. drummondii* cotyledons to differentiate into shoots, directly or via callus, may be attributed to sub-optimal levels of such factors.

Hypocotyl and epicotyl explants from *Sesbania drummondii* were cultured on various combinations of NAA, BA, and IBA. Table 4 shows that epicotyl produced moderate callus that was slow growing. However, hypocotyls had moderate to profuse callusing on the MS medium supplemented with 8.8 μM BA + 1.34 μM NAA; 4.4 μM BA and different concentrations of NAA (1.34 – 8.0 μM). Proliferation of callus was mostly seen at the top of hypocotyls (Fig. 1E). Hypocotyl callus grown on the medium supplemented with NAA and BA was compact and greenish. Epicotyls callused at both ends, and the texture of callus was green and friable. The hypocotyl and epicotyl explants grown on the MS medium supplemented with 2.2 - 6.81 μM BA and 0.24 - 0.49 μM IBA also produced fast-growing callus. On medium containing 2.2 - 6.81 μM BA and 0.24 μM IBA, hypocotyl explants produced scanty roots on the upper part of the explant. The roots on the explants suggest that, hypocotyls have high propensity for root generation in *S. drummondii*. In *Sesbania aculeate*, hypocotyl explants were found to differentiate into shoots directly when cultured on NAA and BA combinations (Bansal and Pandey 1993).

Table 4. Morphogenetic response of hypocotyl and epicotyl explants of *S. drummondii* grown on different concentrations of BA and NAA.

BA [μM]	NAA [μM]	IBA [μM]	Hypocotyl callus [%]	Epicotyl callus [%]
4.4			30	30
8.8	-	-	60	40
9.7	-	-	70	30
8.8	1.34	-	80	60
4.4	2.69		100	100
4.4	5.37	-	90	90
4.4	8.0	-	90	90
2.2		0.24	0	30
4.4	-	0.24	0	30
6.81		0.24	0	30
2.2		0.49	50	50
4.4		0.49	50	50
6.81	-	0.49	30	30

Also in *S. bispinosa* and *S. formosa*, hypocotyls directly differentiated into multiple shoots (Yan-Xiu *et al.* 1993). Studies on *S. drummondii* support the contention of Yan-Xiu *et al.* (1993) that tissue culture response in *Sesbania* species is largely variable and dependent on explant type and age.

Rhizogenesis

Shoots that developed from explants were excised individually and transferred to the MS medium containing different concentration of IBA (0.24 - 2.46 μM) for rooting. Roots were visible within 5 - 10 days following transfer to the rooting medium. After 2-3 weeks, plantlets developed primary and secondary root systems (Fig. 1F). Frequency of rhizogenesis was about 100 %. Increasing concentration of IBA above 0.24 μM had no effect on the frequency of root regeneration. Shoots also produced roots when transferred to a basal medium containing no plant growth regulators, but the intensity of rooting was high with IBA (0.24 - 2.46 μM). A similar response has been reported in *S. formosa, S. sesban* and *S. bispinosa* (Yan-Xiu *et al.* 1993). Shoot elongation was also achieved on the rooting medium. However, faster shoot elongation was recorded on MS medium containing 1.14 μM IAA.

Acclimatization of plantlets

Finally, 7 9 cm long complete plantlets were transferred to pots containing autoclaved peat moss at room temparature where survival of 60 % plantlets was recorded (Fig. 1G). Subsequently, they were transferred to greenhouse conditions where survival rate came down to 30 % after 3 months. One of the reasons for high plant mortality may be improper relative humidity (RH) in greenhouse conditions. Conditions for acclimatization are being optimized by alteration in light and RH conditions.

References

Bansal, Y.K., Pandey, P.: Micropropagation of *Sesbania aculeate* (Pers.) by adventitious organogenesis. Plant Cell Tiss. Org. Cult. **32**: 352-355, 1993.

Detrez, C., Ndiaye, S., Dreyfus, B.: *In vitro* regeneration of the tropical multipurpose leguminous tree *Sesbania grandiflora* from cotyledon explants. - Plant Cell Rep. **14**: 87-93, 1994.

Hanower, J., Cas, G., Bouvet. : Tissue and protoplast culture of *Sesbania rostrata.* - C. R. Acad. Sci. III **303**: 429-434, 1986.

Harris, P.J.C., Puddephat, I.J.: *In vitro* propagation of *Sesbania* species. - Nitrogen Fixing Tree Res. Rep. **7**: 129-131, 1989.

Kapoor, S., Gupta, S.C.: Rapid *in vitro* differentiation of *Sesbania bispinosa* plants – a leguminous shrub. - Plant Cell Tiss. Org. Cult. **7**: 263-268, 1986.

Khattar, S., Mohan Ram, H.Y.: Organogenesis in the cultured tissues of *Sesbania sesban,* a leguminous shrub. - Indian J. Exp. Biol. **19**: 216-219, 1982.

Khattar, S., Mohan Ram, H.Y.: Organogenesis and plantlet formation *in vitro* in *Sesbania grandiflora* (L.) Pers. - Indian J. Exp. Biol. **21**: 251-253, 1983.

Malik, K.A., Saxena, P.K.: TDZ induces high-frequency shoot regeneration in intact seedlings of pea (*Pisum sativum*), chickpea (*Cicer arietinum*), and lentil (*Lens culinaris*). - Aust. J. Plant Physiol. **19**: 731-740, 1992.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassay with tobacco tissue cultures. - Physiol. Plant. **15**: 473-497, 1962.

Murthy, B.N.S., Murch, S.J., Saxena, P.K.: Thidiazuron: a potent regulator of *in vitro* plant morphogenesis. - *In vitro* Cell. Dev. Biol.- Plant **34**: 267-275, 1998.

Pandey, P., Bansal, Y.K.: Plant regeneration from leaf and hypocotyl explants from *Glycine wightii* (W. and A.) Verdc. var longicauda. - Jap. J. Breeding **42**:1-5, 1992.

Powell, R.G., Smith, C.R.: An investigation of the anti-tumor activity of *Sesbania drummondii.* - J. Nat. Prod. **44**: 86-90, 1981.

Powell, R.G., Smith, C.R., Madrigal, R.V.: Antitumor activity of *Sesbania vesicaria*, *S. punicea* and *S. drummondii* seed extracts. - Planta Medica. **30**: 1-8, 1976.

Sahi, S.V., Faison, V., Bryant, N., Andersland, J.: Accumulation of heavy metals by *Sesbania* species. - Proc. 5th International Conference on the Biogeochemistry of Trace Elements, July 11-15, 1999, Vienna, Austria. 582-583, 1999.

Sahi, S.V., Bryant, N.L., Sharma, N.C., Singh, S.R.: Characterization of a lead hyperaccumulator shrub, *Sesbania drummondii.* - Environ. Sci. Technol. **36**: 4676-80, 2002.

Shanker, S., Mohan Ram, H.Y.: Plantlet regeneration from tissue culture of *Sesbania grandiflora.* Curr. Sci. **59:** 39-43, 1990.

Sinha, R.K., Mallick, R.: Plantlets from somatic callus tissue of the woody legume *Sesbania bispinosa* (Jacq.) W.F. Wight. - Plant Cell Rep. **10**: 247-250, 1991.

Vlachova, M., Merz, B.A., Schell, J., De Brujin, F.J.: The tropical legume *Sesbania rostrata*: tissue culture, plant regeneration and infection with *Agrobacterium tumefaciens* and *rhizogens* strains. - Plant Sci. **50**: 213-223, 1987.

Xu Z.H., Yang, L.J., Wai, A., Gao, M.: Plant regeneration in tissue culture of four legumes. Acta Biol. Exp. Sinica **17**: 483-489, 1984.

Yan-Xiu, Z., Dun-Yi., Harris, P.J.C.: Plant regeneration from callus and explants of *Sesbania* spp. Plant Cell Tiss. Org. Cult. **34**: 253-260, 1993.

Chapter III

Genetic transformation of *Sesbania drummondii*

Abstract

Sesbania drummondii is a leguminous shrub mostly distributed in the southeastern parts of the USA. Studies have shown that *Sesbania drummondii* can hyperaccumulate toxic heavy metal such as lead (Pb). This plant is considered a potential source for the phytoremediation of Pb contaminated sites. *Agrobacterium*-mediated transfer of GUS reporter gene was achieved in this species. *Agrobacterium* (K289) containing pCAMBIA 1305.1 plasmid was used for transformation of *Sesbania* explants. Infected explants produced calluses on MS medium containing 0.5 mg/L thiadiazuron. Callus when subjected to GUS histochemical assay produced blue spots demonstrating the expression of the GUS gene. PCR analysis of the genomic DNA from transformed tissue using GUS gene specific primers revealed an amplified fragment of the expected size confirming the presence of the reporter gene. Microscopic sectioning was used to demonstrate the localization of β-glucuronidase inside the cell.

Introduction

Sesbania drummondii has gained considerable attention because of its increasing economic potential. In recent times, this plant has been recognized as a potential source for phytoremediation of heavy metal contaminated sites (Sahi *et al.* 2002). *S. drummondii* has long been recognized as an important medicinal plant, and research has shown efficacy of various therapeutic compounds.

The pharmacological activity of *S. drummondii* seed extracts has been studied providing strong evidence for anti-leukemic activity of these extracts (Powell and Smith. 1981). However, little scientific research has been directed towards understanding the biosynthetic pathways of the several classes of sesbanimides that are responsible for the biological activity of *S. drummondii* extracts.

The transfer of foreign genes into plants has provided new ways to study regulation of development and biosynthetic processes (Koroch *et al.* 2000). Transfer of foreign genes also provides an opportunity to manipulate plants. *Agrobacterium*-mediated transformation is preferred because of its simplicity and efficiency in providing stable integration of transferred DNA into the plant genome.

There are few reports on genetic transformation of *Sesbania* species. Chunhai *et al.* (1991) studied the susceptibility of various *Sesbania* species to infection and callus formation by using wild type *Agrobacterium* strains. Vlachova *et al.* (1987) have demonstrated *A. tumefaciens*-mediated genetic transformation in *S. rostrata* by gall formation near the site of infection. However, genetic transfer in these species has not been proven by histochemical or molecular techniques. There are no reports on genetic transformation of *S. drummondii*, although it could provide the means to understand the mechanism of alkaloid biosynthesis or to improve this crop for phytoremediation purpose. The objective of this work is thus to develop a genetic transformation protocol of *S. drummondii* using *Agrobacterium tumefaciens.*

Materials and Methods

Seed germination

Seeds of *Sesbania drummondii* were scarified using 85 % H_2SO_4 for 30 min, followed by thorough washing under running tap water for 1-h. Seeds were sterilized with 0.2 % $HgCl_2$ and then transferred to magenta boxes containing water–agar (0.6 %) and incubated at 25 – 28 °C under 16-h photoperiod of 20 μmol m^{-2} s^{-1} irradiance provided by cool-white fluorescent tubes.

Explant preparation

Ten-day-old seedlings were harvested for use of explant: cotyledonary node (CN, 1.5 – 2 cm segment of embryonic axis bearing nodes without cotyledons). Explants were surface sterilized with 0.1 % $HgCl_2$ for five minutes and then rinsed three times with sterile deionized water.

Bacterial strains and binary plasmid

Wild type Agrobacterium strain (K289) and K289 strain containing also plasmid (pCAMBIA 1305.1) were used for transformation studies. Plasmid pCAMBIA 1305.1 was procured from CAMBIA, Australia. The binary plasmid carries the *GUS* (beta-glucuronidase) reporter gene and hygromycin-resistance gene (conferring resistance to hygromycin) within left and right border sequences of T-DNA. This plasmid also contains a kanamycin-resistance gene for bacterial selection. The *GUS* gene and hygromycin resistance gene are placed under the control of the 35S CaMV promoter and the NOS + CaMV poly A terminator respectively (Fig. 1).

Preparation of bacterial cultures

A single colony of wild type *Agrobacterium* strain (K289) was used to inoculate 2 ml of liquid MGY media (10 g/L Mannitol, 0.2 g/L KH_2PO4, 2 g/L Na^+ salt of L-Glutamic acid, 0.2 g/L NaCl, 0.2 g/L MgSO4. 7 H_20, 1 g/L yeast extract, DI water, pH 7.0). K289 carrying additional plasmid pCAMBIA 1305.1 was grown in 2 ml MGY medium containing 100 mg/L kanamycin (*Sigma Chemical Co.*). The starter cultures were grown for 18 hrs at 25 °C on an orbital shaker at a speed of 125-RPM to OD_{600} of 0.7 - 0.8 (Turk *et al.* 1994). The cultures were then inoculated into 25 ml fresh media and grown for another 6-8 hrs. Cells were collected by centrifugation at 7000 RPM for 5 minutes at 4 °C and resuspended in 25 mL of liquid MGY media without kanamycin. Acetosyringone (AS) at 0.1 mM concentration was added to the *Agrobacterium* cultures and incubated for 3 hrs to induce the virulence genes in the Ti-Plasmid as described by Mohamalawari *et al.* (2002).

Agrobacterium infection

Before carrying out the transformation studies, a fully-grown two-month-old *Sesbania drummondii* plant was infected using wild type K289 strain to determine its susceptibility to *Agrobacterium* infection.

Infection and culturing of nodal segments

Nodal explants from two-week-old *S. drummondii* plants were excised, sterilized, and co-cultivated with *Agrobacterium* strains K289 pCAMBIA 1305.1 and K289 (wild type), which had been previously induced with AS. These explants were vacuum-infiltrated or inoculated with bacteria into the exposed nodal parts using syringe and hypodermic needle.

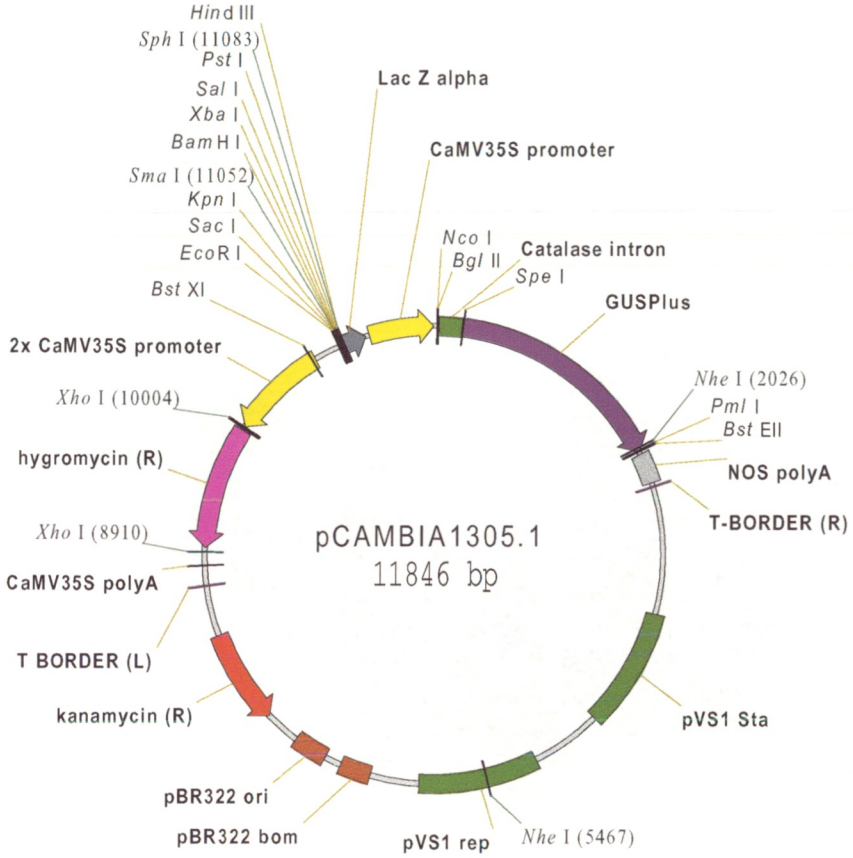


Fig. 1. Schematic representation of plasmid pCAMBIA 1305.1. The left and right border regions enclose a gene that confers resistance to hygromycin and the *GUS* gene driven by the CaMV 35S promoter and NOS poly A terminator.

Inoculated explants were transferred onto solid MS (Murashige and Skoog, 1962) medium containing 2.27 μM of thidiazuron (TDZ) for callus and shoot induction. These cultures were incubated at 25 $\pm$ 2 °C under 16-h photoperiod of 50 µmol m^{-2} s^{-1} irradiance provided by cool-white fluorescent tubes. After 2-3 days the explants were transferred to 0.5 mg/L TDZ containing solid MS medium additionally supplemented with cefatoxine (100 mg/L), carbenicillin (200 mg/L) and vancomycin (100 mg/L) to kill *Agrobacterium.* After 3 and 6 weeks of incubation these cultures were harvested and subjected to *GUS* analysis.

Histochemical analysis of the GUS gene in calli from nodal explants

After 3 and 6 weeks following infection calli and regenerated shoots were cut into 1 cm pieces and incubated in the presence of 50 ml GUS assay buffer containing 25 mg X-Gluc (5-bromo-4-chloro-3-indoly glucuronide); 25 ml of 0.2 M Phosphate buffer; 23.5 ml sterile water; 0.25 ml 0.1 M $K_3[Fe(CN_6)]$; 0.25 ml 0.1 M $K_4[Fe(CN_6)]$. 3H20 and 1 ml Na_2 EDTA (pH 8.0) at 37 °C for 24 hrs to check the expression of *GUS* gene in the putatively transformed tissues (Jeffereson *et al.* 1987). Expression of the *GUS* gene was evaluated by scoring blue spots on the callus and shoot.

DNA extraction and Polymerase Chain Reaction (PCR)

Three and six weeks following infection and co-cultivation of *Sesbania* tissue with K289 and K289 pCAMBIA 1305.1, the plant genome from transformed and non-transformed tissues were extracted and PCR amplified using *GUS* gene specific primers. GUS primers were designed from the *GUS* nucleotides sequences using *Vector Nti 7.0 software* (*Informax Inc*). Plasmid DNA was isolated from wild type *Agrobacterium* and

Agrobacterium bearing the binary plasmid pCAMBIA 1305.1 using the Qiagen plasmid DNA extraction mini-prep kit (Mohamalawari *et al.* 2002). Plant genomic DNA was extracted using the QIAGEN DNAeasy plant genomic DNA extraction mini-prep kit. Genomic DNA was extracted from the blue regions of transformed tissue. The oligonucleotide sequence for the forward GUS primer is 5' TACACCGACCCCGTTTACG 3' and for the reverse primer 5' TTCCACGCTTGCTCACCC 3'. Five μl of (25 ng/μl) genomic DNA, 2x PCR mix containing 1 µl of Taq polymerase (5 u/μl), 2.5 mM dNTPs and 5 μM of both forward and reverse primers were used for PCR amplification. This reaction was carried out in a PTC-100 thermocycler (*MJ Research Inc.*). Amplification conditions were set at 30 cycles for denaturing at 94 °C for 30 sec, annealing at 50 °C for 45 sec, and extension 70 °C for 2 min followed by final extension at 70 °C for 30 min. Completed reactions were stored at 4 °C until analyzed in a 1.5 % agarose gel in the presence of 1x TAE (0.04 M Tris Acetate; 0.001 M EDTA), 0.5 µg of ethidium bromide. The products were run through the gel at 100 V/cm for 1 hr. A picture of the gel was taken using the Kodak gel documentation system to visualize the product and determine its size.

Results and discussion

Agrobacterium-mediated transformation

The fully grown two-month-old *Sesbania drummondii* plant, infected with wild type K289 strain of *Agrobacterium*, developed a gall tumor near the site of the infection after one month (Fig. 2 A). Within T-DNA border sequences there are genes, which encode enzymes required for the biosynthesis of the plant growth regulators auxin and cytokinin. Auxins and cytokinins are responsible for the induction of cell division in the plant cell resulting in callus near the site of infection, called a gall tumor. Gall formation is an indication of plant susceptibility to *Agrobacterium* infection (Koroch *et al.* 2002).

When nodal explants from two-week-old plants were infected with the K289 strain of *Agrobacterium* with and without plasmid pCAMBIA1305.1 and cultured on MS medium containing TDZ, explants produced fast growing calli within two weeks. In some explants, nodes inoculated with *Agrobacterium* developed shoots in a span of three weeks. One of the nodal explants produced callus and embryoid like growth near the site of infection (Fig. 2 B). The *Agrobacterium*-infected nodal explants were cultured on lower concentration of TDZ (2.24 μM), which is considered to be beneficial for the induction of somatic embryogenesis (Malik and Saxena. 1992). Somatic embryogenesis seen in the infected nodal explants may be the outcome of combined effects of growth regulators from *Agrobacterium* T-DNA due to transformation and externally supplied TDZ.

Fig. 2. **A**. *Sesbania drummondii* plant showing gall tumor near the site of wild type K289 *Agrobacterium tumefaciens* infection; **B**. Small embryoid like structures on the nodal explant infected with K289 pCAMBIA 1305.1 and cultured on 2.24 • M TDZ.

Our previous efforts to initiate somatic embryogenesis from callus or from different explants were not successful when using same concentration of TDZ (Cheepala *et al.* 2003). Thus, one can speculate about the role of the auxins and cytokinins expressed in transformed tissue, along with TDZ in inducing somatic embryogenesis in *S. drummondii*.

The histochemical assay for *GUS* gene expression revealed that among the 60 explants used in transformation 35 had blue spots, demonstrating an overall transformation efficiency of 60 % (Table 1). *GUS* gene expression in the transformed callus tissue was observed to be 50 % after three weeks of incubation while 60 % after six weeks. Studies of the *GUS* gene expressing over time indicate that gene expression in *S. drummondii* is relatively stable. Histochemical analyses of GUS gene expression in callus tissue infected with K289 pCAMBIA 1305.1 are presented in Fig. 3 (A and B). Approximately 2 - 4 blue spots were present on each GUS treated explant, and the size of each blue spot ranged from 2 to 3 mm. The blue color in the callus tissue is due to the expression of *GUS* reporter gene. The plasmid pCAMBIA 1305.1 used in the transformation experiments contains the bacterial *GUS* gene inside the T-DNA border sequences driven by the 35 S CaMV promoter (Fig. 1). As a result of transformation, the *GUS* gene was transferred into the plant genome. Expression of *GUS* resulted in the production of beta-glucuronidase, an enzyme that cleaves the substrate X-Gluc. These kinds of results were also shown in *Echnicaea purpurea* leaf explants. In *Echnicea purpurea* transformation, the authors report high frequency GUS expression as a result of *Agrobacterium*-mediated transformation (Koroch *et al* 2002).

Table 1. β-Glucuronidase expression in calli and shoots of nodal explants following infection with *Agrobacterium*. Tissues were tested following three weeks (Wk) or 6 weeks of cultivation.

Agrobacterium strains	Infected explants	Number of explants forming blue spots		Percent of explants forming blue spots	
		3 Wk	6 Wk	3 Wk	6 Wk
K289	60	0/30	0/30	0	0
K289 (pCAMBIA 1305.1)	60	15/30	20/30	50	67

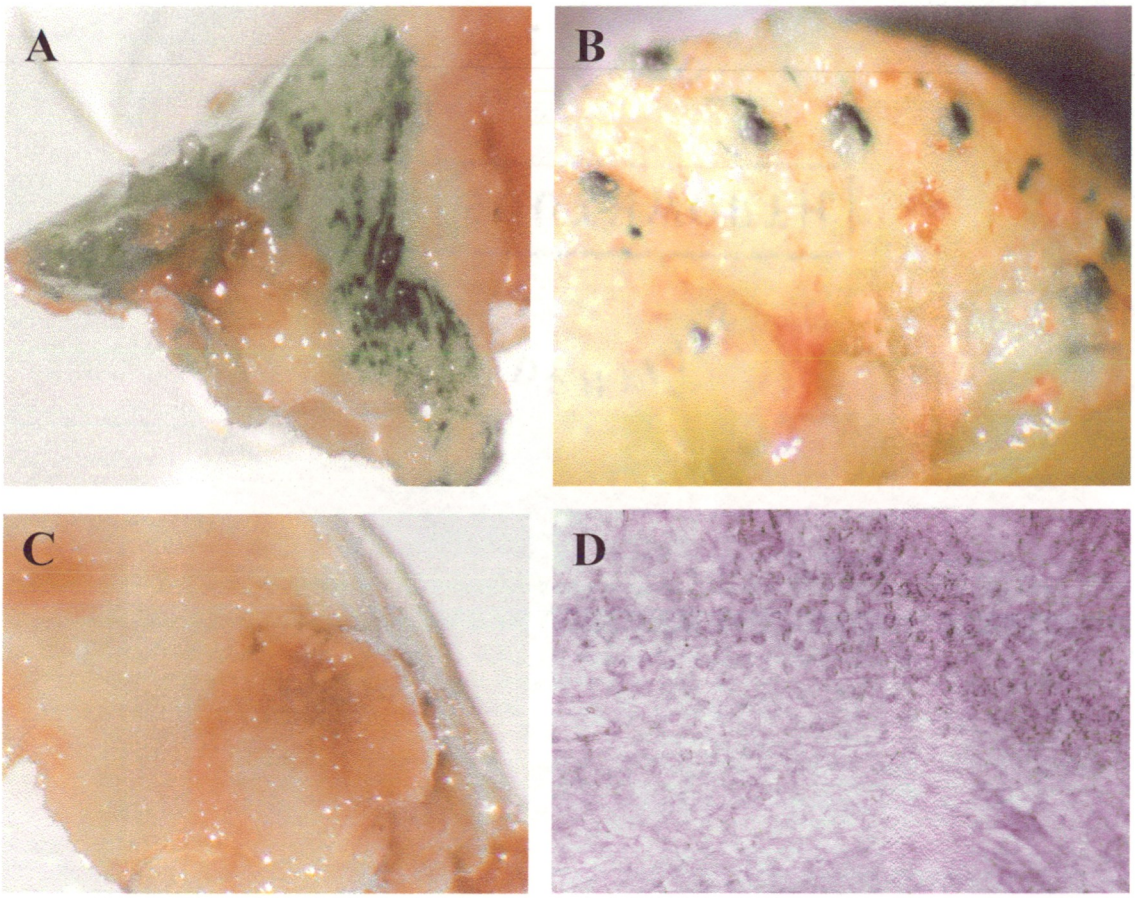


Fig 3. A, B. Expression of the *GUS* gene in callus tissue transformed with *Agrobacterium tumefaciens* strain K289 containing plasmid pCAMBIA 1305.1 and incubated with X-Gluc reagent. **C**. Callus tissue infected with *Agrobacterium* without plasmid pCAMBIA 1305.1 did not develop blue spots when incubated with X-Gluc reagent. **D.** Microscopic section showing intracellular histochemical localization of • -glucuronidase activity in the *Sesbania* callus tissue.

The control *Sesbania* tissue (callus derived from wild type K289 infection) did not show blue color formation when incubated with GUS reagent (Fig. 3 C). Wild type K289 lacks plasmid pCAMBIA1305.1. As a result GUS did not get transferred, β-glucuronidase was not synthesized and substrate X-Gluc could therefore not be cleaved to produce blue color. The absence of blue color also demonstrates the absence of any endogenous *GUS* activity by *Sesbania* itself. To check the possibility of bacterial GUS expression, K289 pCAMBIA 1305.1 and wild type K289 were grown and induced by AS. These bacterial cultures when incubated with GUS reagent at 37 °C for 24 hrs did not show any blue color formation demonstrating the absence of bacterial *GUS* expression.

In order to determine whether *Agrobacterium* with and without plasmid pCAMBIA 1305.1 expressed the *GUS* gene in the presence of *Sesbania* tissue or extract, *Agrobacterium* cultures were grown in macerated *Sesbania* tissues for 12 hrs at 28 °C. These overnight cultures were plated on LB medium and incubated at 28 °C. Following the bacterial growth, GUS reagent was spread on the bacterial colonies and incubated at 37 °C for 24 hrs. None of the bacterial colonies turned blue. These results further demonstrate that blue coloration seen in the transformed *Sesbania* callus tissue was not derived from bacteria present in contact with the *Sesbania* tissues. Moreover, two days following *Agrobacterium* infection, inoculated explants were grown on medium containing various antibiotics (vancomycin, cefatoxine, and, carbenicillin) to kill the *Agrobacterium.* In general, the period required for T-DNA transfer from bacteria to plant cells is 2-3 days (Sahi 1992), Thereby further ruling out the potential for bacterial *GUS* expression in transformed tissues.

Microscopic sectioning of blue calli was also performed to demonstrate where expression of *GUS* was localized. As shown in Fig. 3 D, the blue color is localized inside the cells. Localization of the blue color inside the cell is an evidence for the synthesis of β-glucuronidase enzyme in the cytoplasm of the cells as a result of genetic transformation.

Molecular analysis

To demonstrate the presence of the *GUS* gene in the plant genome, infected tissues were further analyzed by molecular methods. The results from PCR amplification of plasmid DNA of *Agrobacterium* containing pCAMBIA 1305.1, and genomic DNA from both transformed and untransformed plant tissue are shown in Fig. 4. The presence of a similar molecular weight band amplified from *Sesbania* tissue infected with pCAMBIA 1305.1 containing *Agrobacterium* and from isolated pCAMBIA 1305.1 plasmid (lane 2 and 4, Fig. 4) demonstrates that the transformed tissue contains the *GUS* gene. Absence of the band in control tissue transformed with wild-type *Agrobacterium* (lane 1 Fig. 4) demonstrates that the amplified band is indeed derived from the *GUS* gene. It also shows the lack of endogenous *GUS* sequences in the *S. drummondii* calli. The presence of plant catalase intron in the pCAMBIA 1305.1 further ensured that the *GUS* gene is only expressed in plant tissue. Correct translation of *GUS* mRNA is possible after the catalase intron is spliced from the mRNA, which could occur only in the nucleus of eukaryotic cells.

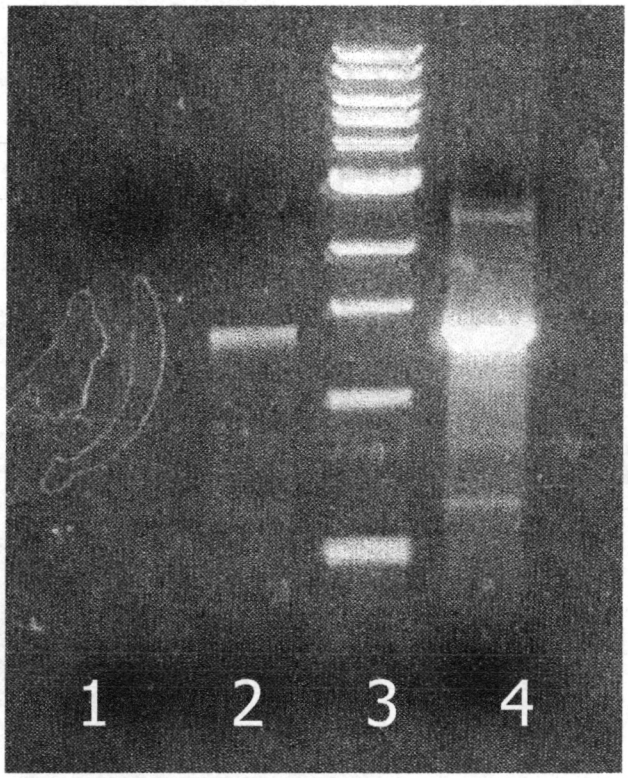


Fig. 4. Presence of *GUS* gene sequences indicated by PCR analysis. Lane 1 represents *S. drummondii* callus tissue transformed with *Agrobacterium* strain K289 (without plasmid). Lane 2 represents callus tissue transformed with *Agrobacterium* strain K289 pCAMBIA 1305.1. Lane 3 contains NEB 1 kb ladder and lane 4 the *GUS* gene amplified from pCAMBIA 1305.1 plasmid.

The earlier studies on transformation of *Sesbania* species were limited to infection and gall formation (Vlachova *et al.* 1987). Chunhai *et al.* (1991) confirmed the transformation in *S. rostrata* by demonstrating the presence of opines in the transformed tissue. Opines are the chemical compounds synthesized from the T-DNA when it becomes integrated into the host plant genome. Thus, the presence of opines in the transformed tissue serves as an indication of transformation (Chunhai *et al.* 1991, Graves and Goldman 1986). However, some of the previous studies by Christou *et al.* (1986) indicate that the presence of excess arginine in the culture medium can also induce the plant to produce opines. Therefore, opine production in transformed tissue may not be used as an only criterion to prove *Agrobacterium*-mediated transformation of plants. In this study various approaches including molecular analysis were used to confirm successful transformation. This study represents the first report on successful *GUS* gene expression in *S. drummondii.*

These initial results on transformation of *S. drummondii* can be utilized for further genetic modification of this species. With the refinement of this transformation technique we can possibly transfer useful genes such as citrate synthase to enhance its potential and efficacy in phytoremediation.

References

Cheepala, S.B., Sharma, N.C., Sahi, S.V.: Rapid *in vitro* regeneration of *Sesbania drummondioi*. – Biol. Plant. In press, 2003.

Christou, P., Platt, S.G., Ackerman, M.C.: Opine synthesis in wild type plant tissue. – Plant Physiol. **82**: 218-221, 1986.

Chunhai, D., Dunyi, Y., Harris, P.J.C.: Genetic transformation of *Sesbania* species with *Agrobacterium tumefaciens*. Nitrogen fixing tree Res. Reports **9**: 134-136, 1991.

Graves, A.C.F., Goldman, S.L. Transformation of Zea *mays* seedling with *Agrobacterium tumefaciens*.- Plant Mol. Biol. **7**: 43-50, 1986.

Jefferson, R.A., Kavanagh. T.A., Bevan, M.W.: GUS fusion β- glucuronidase as a sensitive and versatile gene fusion marker in higher plants. - EMBO J. **6:** 3901-3907, 1987.

Koroch, A., Kapteyn, J., Juliani, H.R., Simon, J.E.: *In vitro* regeneration and *Agrobacterium* transformation of *Echinacea purpurea* leaf explants. Trends in new crops and new uses, 2002. J. Janick and A. Whipkey (eds.). ASHS Press, Alexandria, VA.

Malik, K.A., Saxena, P.K.: TDZ induces high-frequency shoot regeneration in intact seedlings of pea (*Pisum sativum*), chickpea (*Cicer arietinum*), and lentil (*Lens culinaris*). - Aust. J. Plant Physiol. **19**: 731-740, 1992.

Mohamalawari, D., Sharma, N.C., Cristae, P. Sahi, S.V.: Transformation of maize by 2,4,dihydroxy–7-methoxy-2H-1,4-benzoxazin-3(4H)-one resistant *Agrobacterium* strains.- Biotech. Let. **24**: 197-203, 2002.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassay with tobacco tissue cultures. - Physiol. Plant. **15**: 473-497, 1962.

Powell, R.G., Smith C.R.: An investigation of the anti-tumor activity of *Sesbania drummondii.* - J. Nat. Prod. **44**: 86-90, 1981.

Sahi, S.V., Bryant, N.L., Sharma, N.C., Singh, S.R.: Characterization of a lead hyperaccumulator shrub, *Sesbania drummondii.* Environ. Sci. Technol. **36**: 4676-80, 2002

Sahi, S.V.: Effect of DIMBOA, a corn metabolite, on growth and virulence gene induction of *Agrobacterium tumefaciens,* and an attempt to transform corn cells by *Agrobacterium*. - Ph.D thesis, North Carolina State University, 1992.

Turk, S.C., Van Lange, R.P., Regensburg-Tuink, T.J., Hooykaas, P.J.: Localization of the VirA domain involved in acetosyringone-mediated vir gene induction in *Agrobacterium tumefaciens*. Plant Mol. Biol. **25**: 899-207, 1994.

Vlachova, M., Merz, B.A., Schell, J., De Brujin, F.J.: The tropical legume *Sesbania rostrata*: tissue culture, plant regeneration and infection with *Agrobacterium tumefaciens* and *rhizogens* strains. - Plant Sci. **50**: 213-223, 1987.

CHAPTER IV

Concluding Summary

The major goals of this research were to develop (i) *In vitro* regeneration for the medicinally and environmentally important plant *Sesbania drummondii*, and (ii) To develop genetic transformation protocol for *S. drummondii.*

In vitro regeneration

In vitro regeneration of *Sesbania drummondii* was achieved using various nodal explants (cotyledonary and axillary branch node). Nodes were cultured on MS medium containing various concentrations and combinations of cytokinins and auxins for differentiation. Maximum number of shoots was obtained from cotyledonary node on MS medium supplemented with 3.0 mg/l benzyladenine (BA) in a span of four weeks. These explants also produced profuse callus from the basal end on the medium containing 0.5 mg/l thidiazuron (TDZ). Fully grown plants were acclimatized to the green house conditions.

Genetic transformation

Genetic transformation of explants from *Sesbania* was achieved by *Agrobacterium tumefaciens* containing pCAMBIA 1305.1 plasmid. Infected explants were cultured on MS medium containing 0.5 mg/L TDZ for three weeks and six weeks. Expression of the *GUS* gene in callus cells was confirmed by *GUS* histochemical assay. Molecular and microscopic analysis was done to show the presence and expression of *GUS* gene in the transformed tissue.

The developed protocols for regeneration and transformation will be helpful for the production of transgenic *Sesbania* plants with improved efficiency to hyperaccumulate lead, and produce pharmaceutically important compounds.