TISSUE CULTURE OF *CUCUMIS* SPP. AND INTRODUCTION OF CHITINASE GENES INTO *CUCUMIS SATIVUS* CV. ENDEAVOR BY *AGROBACTERIUM TUMEFACIENS*-MEDIATED TRANSFORMATION

by

Simon H.T. Raharjo

B.Sc., Bogor Agricultural University (IPB), Indonesia, 1983

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

in the Department
of
Biological Sciences

SIMON FRASER UNIVERSITY

September 1995

APPROVAL

NAME: Simon H. T. Raharjo

DEGREE: DOCTOR OF PHILOSOPHY

TITLE OF THESIS:

TISSUE CULTURE OF CUCUMIS SPP. AND INTRODUCTION OF CHITINASE GENES INTO CUCUMIS SATIVUS CV. ENDEAVOR BY AGROBACTERIUM TUMEFACIENS-MEDIATED TRANSFORMATION.

Examining Committee:

Chair: Dr. A. Plant, Assistant Professor

Dr. Z. K. Punja, Associate Professor, Senior Supervisor,
Department of Biological Sciences, SFU

Dr. J. M. Webster, Professor
Department of Biological Sciences, SFU

Dr. A. Kermode, Assistant Professor
Department of Biological Sciences, SFU

Dr. G. R. Lister, Associate Professor
Department of Biological Sciences, SFU
Public examiner

Dr. J. E. Carlson, Associate Professor
Biotechnology Laboratory, Faculties of Forestry/Agriculture,UBC
External Examiner

Date Approved OCTOBER 13, 1995

Title of Thesis/Project/Extended Essay

Tissue culture of *Cucumis* spp. and introduction of chitinase genes into *Cucumis sativus* cv. Endeavor by *Agrobacterium tumefaciens*-mediated transformation

Author: (signature)

Simon H. T. Raharjo

(name)

October 13, 1995

(date)

ABSTRACT

Regeneration capacity in tissue culture of cucumber (*Cucumis sativus*) cultivars Endeavor, Calypso and Fidelio on Murashige and Skoog (MS) medium, was tested with different plant growth regulator regimes, explant types and dark incubation, and was found to be dependent upon those factors. Highest frequency of somatic embryo formation (80.0%) was achieved in cultivar Endeavor using petiole explants, 2,4-D/BA (5.0/2.5 μM) medium, and dark incubation. Addition of $AgNO_3$ did not enhance plantlet regeneration while kanamycin at ≥ 40 mg•l^{-1} prevented callus development and somatic embryo formation. In tissue culture of African horned cucumber (*Cucumis metuliferus*), calli were induced from all explant types, using all combinations of auxin (IAA, NAA or 2,4-D) and cytokinin (BA, kinetin or zeatin) tested. However, adventitious buds developed only on petiole explants on medium with NAA/BA or 2,4-D/BA at 2.0/1.0 μM. Upon transfer onto medium with 2.0 μM zeatin, the differentiated calli formed adventitious shoots within 2 to 3 weeks (at a frequency of 14.6%) and later developed into normal plantlets.

Embryogenic suspension cultures of pickling cucumber cv. Endeavor were established in MS medium with 1.0/1.0 μM 2,4-D/BA and remained capable of regeneration after repeated subculture (every 2 weeks) for 3-15 months. When different plating procedures were tested, the greatest frequency of shoot and plantlet regeneration was achieved by plating aggregates onto MS medium with NAA/BA (at 2.0/1.0 or 1.0/1.0 μM). Addition of activated charcoal (0.5%) or $AgNO_3$ (30 μM) in the plating medium did not enhance the frequency of plantlet regeneration.

Pickling cucumber was transformed using *Agrobacterium tumefaciens* strains containing one of three binary vectors with an acidic chitinase gene from petunia, or a basic chitinase gene from tobacco or bean, respectively, driven by the CaMV 35S promoter. The frequency of embryogenic callus formation, following cocultivation of explants and culture on selective medium, ranged from 0 to 12%, depending on strains/vectors used and length of cocultivation. Kanamycin-resistant calli were multiplied in suspension cultures. Transformation by all three vectors using a supervirulent strain was confirmed by PCR amplification of the *NPT II* gene, Southern analysis, and Western analysis for the respective protein. The transgenic plants with the heterologous chitinase genes, and non-transgenic controls, were challenged with fungal pathogens *Colletotrichum lagenarium, Alternaria solani, Botrytis cinerea,* and *Rhizoctonia solani.* There was no significant difference between the transgenic and non-transgenic plants in disease development.

ABBREVIATION LIST

2,4-D = 2,4-dichlorophenoxyacetic acid

2,4,5-T = 2,4-trichlorophenoxyacetic acid

BA = 6-benzyl-aminopurine

CaMV = cauliflower mosaic virus

IAA = indole acetic acid

MS = Murashige and Skoog (1962)

NAA = naphthaleneacetic acid

NPT II = neomycin phosphotransferase II

PCR = polymerase chain reaction

PDA = potato dextrose agar

PGR = plant growth regulator

DEDICATION

My dedication to Ibu and my special thanks to Yuliastuti for patiently looking after Ibu when I am away.

ACKNOWLEDGMENTS

I would like to express my special appreciation to Dr. Zamir Punja for his valuable guidance and supervision in this research, and for encouraging and teaching me to write. My thanks are due to Dr. John Webster and Dr. Allison Kermode for useful advices and critical suggestions of this research project and during the thesis writing. The help of Margarita Hernandez and Dr. Yeyan Zhang in this research work is greatly appreciated. This research and the enhancement of my education at Simon Fraser University were made possible by the Fellowship of the Eastern Indonesia University Development Project - Canadian International Development Agency, as well as by the opportunity given to me to study abroad by the Faculty of Agriculture, Pattimura University, Ambon.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

GENERAL INTRODUCTION

1.1 Botany of *Cucumis* spp.

The genus *Cucumis*, which consists of more than 30 species, belongs to the family Cucurbitaceae Juss (Robinson and Whitaker, 1974; Singh, 1990). The cultivated species include cucumber (*C. sativus* L.), melon (*C. melo* L.), and horned cucumber or kiwano (*C. metuliferus* E. Mey ex. Schrad). These species are annual plants, with large angular leaves, long petioles, simple tendrils, long, branched and hirsute stems, and with climbing or trailing growth habits. Almost all species in the genus *Cucumis* are indigenous to Africa (Robinson and Whitaker, 1974). *C. sativus* is considered to be derived from *C. hardwickii* (Singh, 1990), and both are native of Asia (Robinson and Whitaker, 1974). *C. sativus* was domesticated in India and has been cultivated there for at least 3000 years. It was subsequently distributed to Asia Minor, North Africa, Europe and China (De Ponti, 1985). In India, which is probably the most important gene centre for *C. sativus*, the species is extremely variable in many characters. For instance, many genes for disease resistance can be found in this area (Singh, 1990).

For the most part *C. sativus* is a monoecious species. However, other forms of sex expression, including dioecious, hermaphroditic, male or female forms, may be found. A large percentage of pickling cucumber

cultivars, for example, are gynoecious hybrids (Malepszy, 1988). Cytogenetically, *C. sativus* has a diploid chromosome number of 2n=14. *C. metuliferus* (African horned cucumber) has a chromosome number of 2n=24, and is considered to be more primitive than *C. sativus* (Yadava *et al.*, 1984; Singh, 1990). The fact that *C. sativus* has a chromosome number of 2n=14, which differs from the majority of members of the genus *Cucumis* as well as of the family Cucurbitaceae (Singh, 1990), may explain why this species is usually sexually incompatible with other members of the Cucurbitaceae. For instance, *C. sativus* has been placed alone in one group among four cross-sterile groups in the genus *Cucumis* (Deakin *et al.*, 1971).

The cultivated cucumber is an important vegetable crop in many parts of the world. It grows favourably in areas with temperatures above 20°C, and it has very little or no tolerance to frost. Cucumber production can be found in tropical, subtropical as well as temperate regions. This crop may be grown for commercial purposes, either under field conditions or in controlled environments (greenhouses). In relation to their utilization, cucumber cultivars can be classified as either fresh market (slicing) or pickling types (Lower and Edwards, 1986). The economic importance of the cucumber varies according to its geographic region. This crop is very popular in Europe, North America, USSR and Asia (Malepszy, 1988).

C. metuliferus is resistant to certain viruses and to root knot nematodes (Fassuliotis, 1967; Provvidenti and Robinson, 1974; Punja *et al.*, 1988), and is considered to be a valuable source of disease resistance in cucumber breeding. However, since it is sexually incompatible with other

commercially important *Cucumis* species, including *C. sativus* and *C. melo*, alternative methods for germplasm accession need to be studied.

A great number of cucumber cultivars that have various superior characteristics, such as yield, fruit quality, specific growth habits, and resistance to certain pests and diseases, have been developed by conventional breeding. Their characteristics may vary greatly, inherent with the growing environment, and cultural as well as commercial requirements (Lower and Edwards, 1986). However, this crop is still affected by several major diseases, such as powdery mildew, *Fusarium* wilt, root rots, and some virus diseases (Malepszy, 1988). Transfer of many desirable traits, such as disease resistance, from other species and genera in the Cucurbitaceae through conventional crosses has been difficult due to sexual incompatibility (Kho *et al.*, 1980; Singh, 1990).

1.2 Tissue culture of *C. sativus* and *C. metuliferus*

An important aim of plant tissue culture is the development of the ability to regenerate plantlets or plants from explanted tissues, calli, cells or protoplasts. Regeneration of plants in tissue culture has been achieved in an increasing number of plant species, including cucumber and other members of the family Cucurbitaceae (Wehner *et al.*, 1990). Tissue culture of cucumber has been accomplished from a broad range of cultivars, using a variety of explant sources, culture media, concentrations of plant growth regulators (PGRs), as well as environmental conditions for incubation. Research in tissue culture of cucumber has covered a wide range of

techniques, including organ culture for micropropagation, regeneration from callus cultures via organogenesis or embryogenesis, anther and pollen culture, cell suspension culture, induction of flowers *in vitro*, embryo rescue, and protoplast culture (Handley and Chambliss, 1979; Custers *et al.*, 1988; Malepszy, 1988; Wehner *et al.*, 1990; Chee, 1991b; Punja and Raharjo, 1993).

There is potential usefulness of tissue culture in genetic improvement of cucumber. Since the genetic variability of this crop is limited by sexual incompatibility with other species in Cucurbitaceae (Deakin *et al.*, 1971; Singh, 1990), alternative ways to increase the genetic variability may be pursued via unconventional ways. Tissue culture of cucumber may be useful to achieve variability via somaclonal variation (Moreno and Roig, 1990). For instance, it has been shown in cucumber and other cucurbits that tissue culture may also facilitate selection of certain desirable cell or tissue variants, such as those showing higher resistance to a fungus or a fungal toxin (Malepszy and El-Kazzaz, 1990; Msikita *et al.*, 1990; Mackay *et al.*, 1994), and tolerance to higher concentrations of salt (Baszczyk-Guzek and Szwacka, 1994). Genetic transformation, in which tissue culture has a major role, can be an efficient alternative for introducing variability by bringing foreign genes into the crop (Sarmento *et al.*, 1992). However, an efficient system for plant regeneration is essential for *in vitro* techniques to be useful in achieving these goals. Hence, a majority of recent research has been focused on enhancing regeneration of plantlets from callus and cell cultures, as well as pursuing an efficient transfer of foreign genes into cucumber.

Most studies on cucumber tissue culture use seedlings grown in vitro from seeds as the explant source, although materials from field- or greenhouse-grown plants also have been used. The use of aseptically grown seedlings is preferred, since no surface-sterilization of tissues is needed prior to explant preparation. When aseptic plants are used, inherent contamination problems with explants taken directly from field- or greenhouse-grown plants can be avoided. Diverse parts of plants have been used as starting material for cucumber tissue culture. These include cotyledons, true leaves, hypocotyl segments, petioles, internode segments, root segments, shoot tips, nodal cuttings, immature zygotic embryos, and anthers (Lazarte and Sasser, 1982; Trulson and Shahin, 1986; Ziv and Gadasi, 1986; Custers *et al.*, 1988; Custers and Verstappen, 1989; Kim and Janick, 1989; Cade *et al.*, 1990b; Chee, 1990b; Punja *et al.*, 1990b; Lou and Kako, 1994).

A few studies have been done using tissue culture for micro-propagation of cucumber by multiplication of buds or shoots. Handley and Chambliss (1979) used axillary bud explants to achieve shoot multiplication of the gynoecious cucumber hybrid, Carolina. Low concentrations of auxin and cytokinin, i.e. a combination of 0.1 $mg \cdot l^{-1}$ each of naphthalene acetic acid (NAA) and benzyl aminopurine (BA), were found to be optimal for shoot multiplication. An estimated five-fold increase in the number of plants was obtained by this method compared with the traditional method of rooted cuttings. For a similar purpose, using cucumber hybrids Brumex and Bambina, Sapountzakis and Tsaftaris (1994) used explants of shoot segments with buds and found that BA alone at 0.5 or 1.0 $mg \cdot l^{-1}$ resulted in

a higher frequency of shoot multiplication than if NAA was present in the medium.

An important pre-requisite for the application of plant tissue culture to genetic improvement is the ability to regenerate whole plants from cultured cells, protoplasts or undifferentiated tissue (calli). Plantlet regeneration from somatic tissues of cucumber, as well as from other members of Cucurbitaceae, may be accomplished either through somatic embryogenesis or organogenesis. In somatic embryogenesis, embryos are produced from somatic cells of explants (direct somatic embryogenesis), or are induced on calli that are formed from explant tissues (indirect somatic embryogenesis). In somatic organogenesis, shoots, roots, or other organs are produced on somatic tissues of explants (direct somatic organogenesis), or induced on calli formed by explants (indirect somatic organogenesis) (Donnelly and Vidaver, 1988). The ability of cucumber tissue cultures to undergo somatic embryogenesis, organogenesis, or both, is influenced by various factors, including genotype, explant type, environmental conditions of culture, and the use of plant growth regulators (Bergervoet and Custers, 1986; Trulson and Shahin, 1986; Punja *et al.*, 1990b). For example, it was shown in by Bergervoet and Custers (1986) that direct regeneration of adventitious buds occurred when large hypocotyl explants were cultured on a medium with 0.5 μM IAA and 50 μM kinetin with 24 h of light. In contrast, indirect regeneration of adventitious embryoids via callus formation took place when small hypocotyl explants were cultured on the same medium with 4 μM of each 2,4-D and BA in continuous darkness.

Several studies have investigated plant regeneration from cucumber tissue cultures via somatic organogenesis (Custers and Bergervoet, 1980; Wehner and Locy, 1981; Kim *et al.*, 1988; Gambley and Dodd, 1990). There is a great deal of variation in the capacity for shoot and root formation among different cucumber genotypes (Wehner and Locy, 1981). Using a broad range of cultivars and breeding lines and by single-step culturing on MS medium (Murashige and Skoog, 1962) with 1 $mg \cdot l^{-1}$ of each NAA and BA, Wehner and Locy (1981) found that several genotypes could undergo shoot formation at high frequencies (up to 53%), while in a majority of genotypes they tested, no shoot formation was achieved. Comparing cotyledons and hypocotyls, the former appeared to be a more suitable explant source for regeneration via adventitious shoot formation. Kim *et al.* (1988) found that MS with 0.5 μM 2,4-D and 5 μM BA was the optimal combination for callus growth among various concentrations of auxins (2,4-D and NAA) and cytokinins (BA and kinetin) evaluated. Shoot development was achieved in three cultivars, among 10 they tested, in MS with 0.5 μM NAA and 5 μM BA. No significant difference was observed in the ability of callus tissues derived from different cultivars to undergo root formation. However, shoot differentiation was found to be genotype-dependent. Other investigations showed that age and size of cotyledon explants, as well as concentrations of plant growth regulators added to the medium, also affected adventitious shoot formation (Cade *et al.*, 1990a; Gambley and Dodd, 1990). Using cotyledon explants of cultivar Crystal Salad, Gambley and Dodd (1990) were able to produce as many as 50 shoots per cotyledon by cutting the basal region of the cotyledons before culturing. However, much

of the work on organogenesis has been difficult to repeat (Cade *et al.*, 1990b), and the frequency of regeneration is usually low.

Somatic embryogenesis was first observed in cucurbits by Schroeder (1968). In this study, explants of fleshy pericarp tissues of squash (*Cucurbita pepo*) formed cell clusters which then developed into embryoid structures when cultured on modified Nitsch's medium containing 10 $mg \cdot l^{-1}$ IAA for 18 months or more with periodic subcultures. These cell clusters subsequently developed into embryos which showed polarity and capability of forming shoots and roots. This phenomenon was suggested by Schroeder to be initiation of apomixis that occurred in non-ovulary structures. Somatic embryogenesis was later observed in pumpkin (*C. pepo*) from hypocotyl and cotyledon explants on MS medium containing 1 $mg \cdot l^{-1}$ IBA or 0.3 $mg \cdot l^{-1}$ 2,4-D plus 10% watermelon sap (Jelaska, 1972). Lazarte and Sasser (1982) were probably the first to observe somatic embryogenesis in cucumber. In their study, modified Nitsch's medium with raffinose as a carbon source and containing 0.1 $mg \cdot l^{-1}$ each of BA and GA was used for callus initiation. Embryoids were induced when the calli were transferred onto the same medium with or without kinetin. Further development of embryos and plantlets was achieved when PGRs were removed from the medium.

High frequency of somatic embryogenesis in cucumber was first reported by Malepszy and Nadolska-Orczyk (1983) from calli derived from leaf explants using two-step culturing. The primary medium used was MS containing auxin (2,4-D or 2,4,5-T) and cytokinin (BA or 2-isopentenyl

aminopurine/2iP) at relatively high concentrations and the secondary medium was also MS with a lower level of BA but with no auxin. The explants were covered with yellow gel-like calluses after 4 weeks in the primary medium. When these calluses were transferred to the secondary medium, 6 weeks after initiation, stages of somatic embryo formation proceeded. The somatic embryos could later develop into plantlets.

From the information accumulated so far, it is apparent that the regeneration capacity of cucumber tissue cultures via somatic embryogenesis is influenced by various factors. These factors include genotype, explant source, combination and concentration of PGRs, and environmental conditions of cultures (Trulson and Shahin, 1986; Punja *et al.*, 1990b; Lou and Kako, 1994). For example, to study somatic embryogenesis involving a broad range of cultivars and breeding lines, Trulson and Shahin (1986) employed two types of media to optimize somatic embryo development. They used MS supplemented with a combination of 1 $mg \cdot l^{-1}$ 2,4-D, 1 $mg \cdot l^{-1}$ NAA and 0.5 $mg \cdot l^{-1}$ BA for induction of calli and somatic embryos. The same medium, but lacking 2,4-D, was used for embryo maturation. Results from this study showed that the capacity of explants or explant-derived calli to produce somatic embryos, as indicated by percentage of explants producing somatic embryos or by number of embryos formed per explant, was influenced by genotype. The range of regeneration frequency was from 8% (in the line W744GP) to 100% (in the cultivar Bush Slicer).

Using cultivars Gy 14A, Straight-8 and Sumter, a study by Cade *et al.* (1990b) showed that regeneration capacity via somatic embryogenesis was dependent upon genotype and concentration of auxin used for somatic embryo induction and maturation. By employing two-step culturing, in which tissues were removed from the initiation medium containing 2,4-D plus kinetin after 3 weeks and transferred to maturation medium containing 1 $mg \cdot l^{-1}$ NAA and 0.5 $mg \cdot l^{-1}$ kinetin for 3 weeks, more plantlets were obtained than if single-step culturing was employed. In another study, combination of 2 $mg \cdot l^{-1}$ 2,4-D and 0.5 $mg \cdot l^{-1}$ kinetin in the initiation medium, and a combination of 1 $mg \cdot l^{-1}$ NAA and 0.5 $mg \cdot l^{-1}$ BA in the embryo maturation medium, was also optimum for somatic embryogenesis in cultivar Pointsett 76 (Chee, 1990b).

In a study which involved a broad genotype range of members of the genus *Cucumis*, including *C. sativus* var. *sativus*, *C. sativus* var. *hardwickii*, *C. melo* and *C. metuliferus* (Punja *et al.*, 1990b), not only genotype and PGR regime, but also dark incubation and explant source, influenced regeneration capacity, via somatic embryogenesis and/or organogenesis. Among combinations of auxins (2,4-D, NAA and IAA) and cytokinins (BA, kinetin and zeatin) added to MS medium, a combination of 2,4-D and BA, each at 5 μM, was the most suitable for induction of somatic embryogenesis. Incubation in the dark for 2-3 weeks resulted in a higher frequency of regeneration, inclusive of somatic organogenesis, than if cultures were incubated entirely in the light. When cotyledon, true leaf and petiole explants were compared, the use of petiole explants resulted in the highest regeneration frequencies (Punja *et al.*, 1990b).

Callus can be induced from *C. metuliferus* with ease using almost all combinations of an auxin and a cytokinin (Punja *et al.*, 1990b). However, no differentiation was observed, except at a low frequency when a combination of IAA and BA was added to the medium. Plantlets were not obtained in this study.

1.3 Cell Suspension Culture of *C. sativus*

At least four previous studies involving plantlet regeneration from cell suspension cultures of several fresh market cultivars of cucumber have been published (Bergervoet *et al.*, 1989; Malepszy and Solarek, 1986; Ziv and Gadasi, 1986; Chee and Tricoli, 1988). In general, suspension cultures have been initiated from calli which are putatively embryogenic and regeneration of plantlets is achieved by plating the aggregates from the suspension cultures onto solid medium.

Chee and Tricoli (1988) induced formation of calli, for initiation of suspension cultures, by incubating leaf explants of the cultivar Poinsett 76 on MS medium containing 5 μM 2,4,5-T and 4 μM BA, in the dark for 3 weeks, followed by transfer to the light and subcultures at 3-week intervals. Suspension cultures were initiated by adding 2 g of embryogenic callus in 25 ml of liquid MS medium containing the same concentrations of 2,4,5-T and BA, and shaking at 125 rotaries per minute (rpm). They were kept at 26°C and a photoperiod of 16 $h \cdot d^{-1}$. A high frequency of regeneration of normal plantlets was achieved from cell suspension cultures via embryogenesis by washing the cells with MS medium

containing activated charcoal prior to embryo development in liquid MS basal medium, and later plating onto solid conversion medium (MS basal medium).

In a study by Bergervoet *et al* (1989) using the cultivar Hokus, calli for initiation of suspension cultures were produced from primary leaves incubated on MS medium containing 2,4-D/BA (5/5 μM). Suspension cultures were maintained continuously in liquid MS medium containing 2,4-D/BA (5/5 μM), with subcultures once a week. Alternatively, the suspension cultures were maintained by three subcultures (once a week) in liquid MS medium with NAA/BA (5/5 μM), and a fourth subculture in medium with 2,4-D/BA (5/5 μM). When suspension aggregates in the medium with NAA and BA were plated onto solid medium containing a low concentration of IAA and kinetin, a high regeneration frequency of normal plantlets was achieved through bud formation (organogenesis) (Bergervoet *et al.*, 1989). In another study, plantlet regeneration via embryogenesis was attained using a liquid/solid double layer system containing charcoal (Ziv and Gadasi, 1986).

1.4 The Effects of $AgNO_3$ on Cucumber Tissue Culture

The addition of silver, mostly in the form of $AgNO_3$, to the tissue culture medium has been shown to enhance plantlet regeneration of diverse plant species, such as *C. melo* (Roustan *et al.*, 1992), *Brassica campestris* (Palmer, 1992), *Nicotiana flambaginifolia, Triticum aestivum* (Purnhauser *et al*, 1987) and *Zea mays* (Songstad et al., 1991). As well,

plantlet regeneration from protoplast cultures of *Brassica juncea* and *B. campestris* is enhanced by adding the chemical (Pua, 1990; Pauk *et al.*, 1991). In the presence of 1 to 10 $mg \cdot l^{-1}$ $AgNO_3$ in the culture medium, regenerable cultures of Brussels sprouts (*Brassica oleracea* var. *gemmifera*) could be maintained for a much longer period of time than if $AgNO_3$ was absent (Williams *et al.*, 1990). $AgNO_3$ has been shown also to be useful in *Agrobacterium*-mediated transformation of some plant species. For example, it was reported to be required to achieve regeneration from transformed tissues of *B. campestris* (Mukhopadhyay *et al.*, 1992), and was necessary to enhance regeneration efficiency in *Arabidopsis thaliana* (Marton and Browse, 1991) and *Solanum tuberosum* (De Block, 1988; Higgins *et al.*, 1992).

The enhancement of plantlet regeneration in tissue cultures by the presence of $AgNO_3$ is related to its role in the inhibition of the effect of ethylene (Roustan *et al.*, 1992) which is sometimes produced by cultured tissues and may accumulate in culture containers (Lentini *et al.*, 1989; Roustan *et al.*, 1992). However, the mechanism by which the presence of ethylene affects, usually adversely, somatic embryogenesis or organogenesis is not yet well-understood.

1.5 An Overview of *Agrobacterium*-mediated Transformation

Agrobacterium, particularly strains of *A. tumefaciens*, have proven to be excellent vectors for transferring foreign genes into plant cells. The genus consists of gram-negative soil bacteria. Naturally, *A. tumefaciens*

causes crown gall disease in a wide range of plant species (Hooykaas and Schilperoort, 1992). The capability of *A. tumefaciens* to induce galls (tumours) is due to the presence of a large Ti (tumor-inducing) plasmid, which is a circular, double-stranded DNA molecule of about 200 kb organized into several genes. A natural Ti-plasmid has four important regions: (a) transferred DNA (T-DNA) region, (b) region of origin of replication, (c) plasmid conjugation region, and (d) virulence (*vir*) region. In addition, it carries genes for catabolism of opine(s) (Draper and Scott, 1991). *A. tumefaciens* has developed mechanisms for the transfer and integration of the T-DNA segment of the Ti-plasmid into the plant nuclear genome (Hooykaas and Schilperoort, 1992). A clearer picture of the molecular mechanisms involving the transformation of plants by *Agrobacterium* has been described (e.g. Zambryski *et al.*, 1989; Hooykaas and Schilperoort, 1992; Zambryski, 1992) and the mechanisms have been routinely utilized to transfer cloned, foreign genes into plants.

The T-DNA contains two functionally important sequences, the oncogenes and the genes for biosynthesis of opine(s) (Hooykaas and Schilperoort, 1992). The oncogenes code for the biosynthesis of an auxin (i.e. indole-3-acetic acid) and a cytokinin (i.e. zeatin riboxide or isopentenyl adenosine 5'-monophosphate) and are responsible for the induction and maintenance of cell division in neoplastic outgrowth of tumour tissues. Opines are amino acid derivatives and a source of carbon and nitrogen for the bacterium. Based on the production of this opine, *A. tumefaciens* strains may fall into one of four types: nopaline, octopine, leucinopine and succinamopine (Melchers and Hooykaas, 1987). Nopaline strains often

have wider host ranges and are more virulent than the octopine ones. Leucinopine and succinamopine strains show a strong capability of transforming plants and are therefore considered to be supervirulent strains (Komari, 1989).

Two regions in the Ti plasmid are essential for the T-DNA transmission process: the border sequences of T-DNA, especially the right border, and the *vir* region. Left and right borders flanking the two ends of the T-DNA consist of 25-bp direct repeat DNA sequences. The border sequences act in a *cis* and polar fashion, and have a role in the recognition of specific endonucleases functioning in excision of the T-DNA (Wang *et al.*, 1984). A set of genes in the *vir* region plays a role in mediating the transfer of T-DNA to cells of the host plant. The genes are not themselves transferred into plant cells. Acting in *trans*, however, they mediate the process of the excision and transfer (Horsch *et al.*, 1988; Draper and Scott, 1991).

During the initiation of *Agrobacterium* colonization, the bacterial cells attach to the wounded plant cells, mediated by expression of bacterial chromosomal genes, *chvA* and *chvB*. Activation of *vir* genes is induced by phenolic compounds in plant wound exudates, such as acetosyringone (4-acethyl-2,6-dimetoxyphenol) and α-hydroxyacetosyringone (Binns and Thomashow, 1988). Two *vir* genes, *virA* and *virG*, are constitutively expressed and involved in mediating the induction of the other *vir* genes (Stachel and Zambryski, 1986). *VirA* product is a membrane-associated protein and probably a chemoreceptor or a transport protein for

acetosyringone (Winans *et al.*, 1989). *VirG* is regulated in a complex way and encodes a positive regulatory protein. In association with signal molecules, this protein activates the expression of other *vir* genes. These processes lead to excision of the T-DNA, export of the T-strand from bacterial cells to the plant nucleus, and finally the integration into the plant genome (Zambryski *et al.*, 1989). Within the cells of tumour tissues, genes carried in T-DNA are expressed, which are responsible for oncogenicity (Draper and Scott, 1991). Thus, by transferring the set of genes in T-DNA, *Agrobacterium* induces oncogenicity and exploits the plant's metabolism in order to produce opines which are not used by the plant but upon which the bacterium is nourished.

The oncogenes, which are not essential for T-DNA transfer, can be removed without interfering with the capability of *Agrobacterium* to transfer DNA into the plant genome. The replacement of oncogenes with chimerical genes constitutes the basis for *Agrobacterium*-mediated plant transformation (Klee *et al.*, 1987). The disarmed and engineered bacterial plasmid will not cause tumours on host plants. Foreign genes which are to be introduced into plant cells, such as genes for a selectable marker (e.g. antibiotic resistance) or genes with certain agronomic importance, can be inserted in the positions within T-DNA formerly occupied by the oncogenes (Draper *et al.*, 1988). Thus, by cloning foreign DNA into the T-DNA, it is possible to exploit the natural ability of *Agrobacterium* to transfer foreign DNA into the plant genome.

Vector systems based on the use of Ti-plasmids for gene transfer may fall into either of two categories: co-integrative system and binary system (Walden, 1988; Draper and Scott, 1991). In a co-integrative system, foreign genes are integrated within the T-DNA of a resident Ti-plasmid via a homologous recombination. In a binary system, an *Agrobacterium* strain carries a deleted Ti-plasmid (without T-DNA region) that provides the *vir* functions in *trans* and a separate vector that provides the genetically engineered T-DNA. The advances in the development of the binary system have been resulted from the fact that the T-DNA and the *vir* regions can be separated into two different plasmids without the loss of DNA transfer capability (Hoekema *et al.*, 1983). Binary vectors are constructed using a wide-host-range plasmid which can replicate in both *E. coli* and *Agrobacterium* cells. To function properly for plant genetic transformation, a binary vector generally contains several important sequences: (a) border sequences of T-DNA for successful transfer of DNA from *Agrobacterium* cells to the plant genome, (b) a selectable dominant marker under the control of plant transcription signals, (c) a single replicon and markers for maintenance and selection in *Agrobacterium* and *E. coli*, and (d) restriction sites suitably positioned to allow the cloning of desired foreign sequences and their detection (Miki and Iyer, 1990). The desired foreign gene is inserted within the engineered T-DNA.

To transfer DNA into plants using *A. tumefaciens* Ti-plasmids, the plant materials are infected with a bacterium strain that carries the engineered vector system. Various types of tissue or cell sources, including tissue sections, calli, cell clumps, or protoplasts may be used in

A. tumefaciens-mediated transformation (Draper *et al.*, 1988). For successful production of transgenic plants, an efficient gene transfer into cells, both competent for integrative transformation as well as for regeneration, is required (Potrykus, 1990). The important requirement to achieve competence for transformation using intact plant tissues is wounding and thus release of acetosyringone. Finally, the cells of tissues that have been transformed must be able to divide and finally differentiate into organs or whole plants.

Leaf disc inoculation (Horsch *et al.*, 1985) is a commonly used technique in *A. tumefaciens*-mediated transformation. This technique involves sectioning sterile leaves, inoculating with *Agrobacterium* and incubating (cocultivation) for a certain length of time. After the cocultivation step, the transformed cells are allowed to proliferate on a medium which contains a selective chemical, such as kanamycin, resistance to which is encoded by the selectable marker gene in the T-DNA segment of the vectors used for the transformation. Putatively transformed plants may be regenerated through somatic embryogenesis or organogenesis. As a variation to this technique, depending on the plant species or specific purposes, and inherent with the ease for plant regeneration in tissue culture, other types of tissues may be used as explants, including cotyledons, hypocotyls, stems, root, etc. (Draper and Scott, 1991; Gheysen *et al.*, 1992).

Confirmation of putatively transformed tissues or whole plants may be obtained through several ways, including: (a) phenotypic assay, (b)

enzymatic assay, (c) genomic DNA assay, (d) Western blot analysis, and (e) progeny analysis (Horsch *et al.*, 1985; Walden, 1988).

Phenotypic assay relies on the observation of phenotypic expression resulting from the presence of the transgene product(s) in the transformed plants. This can be a plant with the ability to grow under selection, e.g. in the presence of kanamycin in the medium. If the transgene product is a enzyme, enzymatic assays may be performed to determine the presence and level of activity of the enzyme in the transformed plant or tissue.

Confirmation of the presence and integration of the foreign DNA in the plant genome can be carried out by two techniques: Southern blot and Polymerase Chain Reaction (PCR) analyses. There are two approaches when using Southern Blot analysis. In the first approach, genomic DNA is digested with an endonuclease which cuts twice and excises a fragment within the inserted DNA to yield a band with a certain size, such as a 2.2-kb band for the *NPT II* fragment (Chee, 1990a; Sarmento *et al.*, 1992). With the second approach, endonuclease digestion is carried out using an enzyme that cuts the DNA once outside the inserted T-DNA region. This yields hybridizing band(s) indicative of the location of the inserted fragment in the plant genome and gives an assessment of minimal copy numbers of the inserted DNA. The use of PCR for confirmation of transformation is based on amplification and detection of specific inserted sequences in the transformed plants. This relies on the use of oligonucleotide primer sequences which are synthesized for amplification of specific genes, such as *NPT II* and *GUS* genes (Chee, 1990a).

Assays for expression of the introduced gene may be performed through detection of the gene product by Western blot analysis or by other standard immunological methods using a suitable antibody (Lichtenstein and Draper, 1985)

1.6 *Agrobacterium*-mediated Transformation of Cucumber

One of the most important applications of cucumber tissue and cell culture is for genetic improvement through genetic engineering (Trulson *et al.*, 1986; Punja *et al.*, 1990b); however, this avenue has been explored only recently. A few studies on transformation of cucumber using *Agrobacterium* have been reported; most of them deal with the transfer of a gene for kanamycin resistance (neomycin phosphotransferase II; *NPT II*) into fresh market cucumber. Trulson *at al.* (1986) transformed cucumber cv. Straight 8 using *A. rhizogenes* with a binary system which consisted of the Ri-plasmid and a binary vector derived from the Ti-plasmid of *A. tumefaciens*. Transformation was conducted by smear-inoculation of hypocotyl explants with bacterial inoculum and cocultivation for 1 week. Transgenic plants containing the *NPT II* gene, and showing resistance to kanamycin, were recovered after regeneration of plantlets from roots which developed from the surfaces of inoculated hypocotyls. In other studies which used *A. rhizogenes* (Van der Mark *et al.*, 1989; McInnes *et al.*, 1991), roots induced on hypocotyl or cotyledon explants after infection with the bacterium exhibited the transformed phenotype, i.e. synthesis of opine.

However, no regeneration of transgenic plants were achieved in these studies.

Chee (1990a) accomplished successful transformation of cucumber cultivar Poinsett-76 using an *A. tumefaciens* binary system carrying an *NPT II* gene. In this study, cotyledon explants were cocultivated with *Agrobacterium* for 4 d. The calli that developed were selected on initiation medium with 100 $mg \cdot l^{-1}$ kanamycin, and plantlet regeneration was achieved via somatic embryogenesis (Chee, 1990a). All genomic DNAs isolated from R_0 and R_1 plants that were resistant to kanamycin were proven to contain the *NPT II* gene. Sarmento *et al.* (1992) described various factors influencing *A. tumefaciens*-mediated transformation of pickling cucumber. In this study, sizes of leaf and petiole explants, bacterial concentration for inoculation, and length of cocultivation, all influenced the efficiency of transformation. Use of a tobacco feeder layer and addition of acetosyringone also were studied and were found to enhance the overall efficiency. Southern analysis showed multiple integration of the transferred gene (the *NPT II* gene) in R_0 and most of the R1 transgenic plants. In a similar study by Hammar and Grumet (1990), it was shown that the optimal conditions for transformation included the use of 45 $mg \cdot l^{-1}$ kanamycin in the selection medium, 10-minute inoculation, and a cocultivation for 3 d. In addition the *NPT II* gene, the *GUS* reporter gene has been used in *A. tumefaciens*-mediated transformation of cucumber (Colijn-Hooymans and Hakkert, 1991; Ladyman and Forsman, 1991) to monitor the DNA transfer. The study by Colijn-Hooymans and Hakkert (1991) suggested that the differences in the regeneration capacity of three

genotypes they used was reflected in the competence of the explant sources to develop into transgenic plants.

Using procedures previously developed (Chee, 1990a), Chee and Slightom (1991) successfully transferred the cucumber mosaic virus (CMV) coat protein gene into cucumber cv. Poinsett 76. A binary vector used in this study contained *NPT II* and *GUS* genes, in addition to CMV coat protein gene. Immunological detection confirmed the expression of the CMV coat protein in R_1 transgenic plants. A field test was conducted using progenies from a cross between cultivar Straight 8, a susceptible cultivar, and the transgenic plants expressing the CMV coat protein. The results showed a delay in the onset of CMV infection as compared to the control plants (Slightom *et al.*, 1990). The transformation of cucumber with CMV coat protein gene represents the first reported successful transfer of an agronomically useful gene into fresh market cucumber. There are no other reports of the introduction of genes into pickling cucumber cultivars.

1.7 Chitinases and Their Use in Plant Genetic Engineering

1.7.1 Plant chitinases and their properties

Plants have developed mechanisms for protection against potential pathogen attack. These involve alteration of protein synthesis and production of enzymes that can inhibit the growth of invading parasites (Verburg and Huynh, 1991). In addition to the development of resistant morphological features, the development of disease resistance in plants is usually expressed by the accumulation of host-synthesized polypeptides that

are produced in response to pathogen attack. Among these are: a) enzymes involved in the synthesis of phytoalexins, b) enzymes leading to the formation of physical barriers to fungal invasion, c) inhibitors of pathogen enzymes, and d) lytic enzymes. As a group, they are called pathogenesis-related (PR) proteins (Broglie *et al.*, 1986; Boller, 1988).

Chitinases (poly (1,4-(*N*-acetyl-β-D-glucosaminide) glucanohydrolase, EC 3.2.1.14) belong to the lytic enzymes which are capable of hydrolyzing chitin (a β-1,4-linked polymer of N-acetylglucosamine), which is a major component of the cell walls of many fungi (Graham and Sticklen, 1994; Meins Jr *et al.*, 1994). Chitinases are believed to have a defence role in plants, based on circumstantial evidence. First, purified chitinases show antifungal activity (Huynh *et al.*, 1992; Park *et al.*, 1992; Swegle *et al.*, 1992), are able to cause hyphal tips of fungi to lyse *in vitro* (Benhamou *et al.*, 1993; Sela-Buurlage *et al.*, 1993), and they inhibit fungal growth (Schlumbaum *et al.*, 1986). Second, in healthy plants, chitinases are usually expressed at a very low level but they are usually induced at a higher level in response to pathogen attack (Metraux, 1986; Meins Jr and Ahl, 1989; Rousseau-Limouzin and Fritig, 1991). Third, chitin does not occur in plants but is an important component of fungal cell walls (Metraux, 1986).

More than 40 higher plants, members of both dicotyledonous aand monocotyledonous species, have been reported to produce chitinases (Punja and Zhang, 1993). Chitinases can be found in different organs and tissues of plants, including embryos, seeds, cotyledons, leaves, stems, flower and roots, as well as in cultured calli, cells and protoplasts (Punja and Zhang, 1993). Some microorganisms, such as the bacterium *Serratia*

marcescens and fungi such as *Aspergillus* and *Trichoderma*, produce this type of enzyme (Dunsmuir and Suslow, 1989; Shapira *et al.*, 1989; Vasseur *et al.*, 1990).

Molecular and biochemical properties of plant chitinases have been extensively studied (e.g. reviewed by Boller, 1988; Graham and Sticklen, 1994). Chitinases may be characterized based on their molecular weight, acid-solubility, extracellular or intracellular localization, and induction by pathogens (Metraux *et al.*, 1989). The enzymes, which usually have a molecular weight of 25-36 kD, are hydrolases; they catalyze the hydrolysis of β-1,4-N-acetylglucosamine linkages of chitin polymers. Almost all plant chitinases are endochitinases that degrade chitin from within the polymer rather than from its terminus, and they release chitooligosaccharides (oligomers of β-1,4-N-acetylglucosamine) of various lengths from chitin (Graham and Sticklen, 1994). Thus, differing from prokaryotic and fungal chitinases which usually degrade chitin completely, most plant chitinases hydrolyze chitin partially (Boller, 1988; Kombrink *et al.*, 1988; Mauch and Staehelin, 1989). Some plant chitinases have been found to also have lysozyme activity in that they hydrolyze peptidoglycan present in bacterial cell walls (Boller *et al.*, 1983; Majeau *et al.*, 1990; Park *et al.*, 1992).

A single species of plant may produce several isoforms of chitinases, as shown in cucumber (Majeau *et al.*, 1990; Zhang and Punja, 1994), tobacco (Sela-Buurlage *et al.*, 1993), pea (Mauch and Staehelin, 1989) and barley (Kragh *et al.*, 1991). Chitinases exist in isoforms exhibiting either acidic or basic isoelectric points (Boller, 1988; Keefe *et al.*, 1990; Lawton *et al.*, 1992). Both forms have been implicated in the induced response of plants to

infection by fungal pathogens. It has been shown that chitinases are encoded by a small family of genes (Boller, 1988; Davis *et al.*, 1991; Nishizawa and Hibi, 1991; Rasmussen *et al.*, 1992a).

Based on biochemical and molecular characteristics, chitinases have been classified into at least six classes (Meins Jr *et al.*, 1994). The first three classes have been grouped on the basis of amino acid sequence similarity to initially classified tobacco chitinases (Shinshi *et al.*, 1990). Class I chitinases have a highly conserved cysteine-rich catalytic domain which consists of about 40 amino acids in the N-terminus, and a catalytic domain, separated by a variable hinge region. The C-terminus has a 7-amino-acid extension which plays a role in targeting the protein into the vacuole (Neuhaus *et al.*, 1991a). Class I chitinases are usually basic and vacuolar. Several acidic chitinases which have similar amino acid sequences to those of class I have been considered to be in a subclass of the class I (Punja and Zhang, 1993). Class II chitinases have similar amino acid sequence (60-64% homology) to class I, but lack a cysteine-rich N-terminus, are generally acidic and are targeted extracellularly. Class III chitinases have a different amino acid sequence in the catalytic domain from classes I and II, lack the cysteine-rich domain, and may be acidic or basic. Other chitinases which are similar to class I chitinases, but smaller in size because of some deletions in the cysteine-rich and catalytic domains and lack the C-terminal extension, are placed into class IV (Collinge et al., 1993; Punja and Zhang, 1993). Class V chitinases have an amino acid sequence similar to that of the stinging nettle (*Urtica dioica*) lectin precursor and have a duplicated N-terminal lectin domain. Class VI chitinases have an amino acid sequence similar to those

of the bacterial exochitinases from *Baçillus circulans, S. marcescens,* and *Streptomyces plicatus,* but different from those of the class I-IV chitinases (Meins Jr *et al.,* 1994).

Chitinases, as well as β-1,3-glucanases, usually accumulate in the outer tissues of plants (Keefe *et al.,* 1990). However, subcellular localizations of basic and acidic chitinases differ. Basic forms are mainly localized in the vacuoles of plant cells, whereas the acidic ones occur extracellularly or are secreted into the extracellular space (Boller and Vogeli, 1984; Legrand *et al.,* 1987; Mauch and Staehelin, 1989; Keefe *et al.,* 1990; Samac *et al.,* 1990).

1.7.2 Role of chitinases in plant defence against pathogens

Chitinases alone, or in combination with β-1,3-glucanases, have been shown to exhibit pronounced antifungal activity (Schlumbaum *et al.,* 1986; Huynh *et al.,* 1992). Chitinase activity is locally increased in many plants infected by fungal pathogens (Metraux, 1986; Schlumbaum *et al.,* 1986; Punja and Zhang, 1993). To inhibit fungal growth and infection, chitinases may function in various ways, which include: (a) degradation of fungal or bacterial cell walls, (b) inhibition of fungal growth, and (c) release of elicitors that may induce other mechanisms of plant resistance (Boller, 1988).

It has been demonstrated that purified plant chitinases readily liberate chitin oligomers (chitooligosaccharides) from purified fungal cell walls (Boller *et al.,* 1983; Schlumbaum *et al.,* 1986). Schlumbaum *et al.* (1986) demonstrated that plant chitinases were highly potent growth inhibitors of *Trichoderma viride* (a non-pathogenic test fungus). Chitinases

enzymatically attacked newly formed chitin and inhibited the growth of fungal hyphal tips. Chitinase alone, at as low as 2 $\mu g \cdot ml^{-1}$, was sufficient to inhibit growth of *T. viride in vitro*. Chitinase alone sometimes does not inhibit growth of some fungi, but combinations of chitinase and β-1,3-glucanase strongly inhibited fungal growth (Mauch and Staehelin, 1989; Sela-Buurlage *et al.*, 1993). Because tips of many fungal hyphae contain both chitin and glucan, the presence of both enzymes might cause synergistic inhibitory effects. In addition, products released from the degradation of fungal cell walls may serve as fungal elicitors for plant defence reactions (Kurosaki *et al.*, 1988).

Acidic chitinases may function differently from the basic forms during pathogen attack. Extracellular (acidic) forms are thought to have an early defence function. This may be by a direct fungicidal action on hyphae invading intercellular spaces and possibly an indirect action by causing release of a fungal elicitor which results in a hypersensitive reaction of the host (Boller, 1988; Mauch and Staehelin, 1989). The vacuolar (basic) forms may function later when cell breakdown releases the vacuolar contents into the extracellular spaces (Keefe *et al.*, 1990).

Whether chitinase activity is induced locally or systemically is still a controversy. Chitinase activity is usually very low in uninfected plants (Graham and Sticklen, 1994). Metraux (1986) demonstrated that infection of the first primary leaves of cucumber with pathogens (*Colletotrichum lagenarium, Pseudoperonospora cubensis, Pseudomonas lachrimans,* and TMV) caused a strong increase in chitinase activity, ranging from 60 to 2,000-fold, depending on the pathogen used. Induction of chitinase was

highest at sites of pathogen attack but also occurred within other leaves. They suggested that the induction of chitinase was an element of both local and systemic response. In contrast, Meins Jr and Ahl (1989) and Rasmussen *et al.* (1992b) found that increases of chitinase were confined to infected parts of the plants they tested, i.e. tobacco and canola (*Brassica napus*), respectively, indicating that this response was local rather that systemic.

1.7.3 Expression of chitinase genes in transgenic plants

One of the possible ways to improve disease resistance in transgenic plants involves the expression of genes coding for PR proteins (Cornelissen and Melchers, 1993; Kamoun and Kado, 1993). Several genes related to disease resistance, including those coding for chitinases, virus coat proteins, virus satellite RNA, and phaseolin (a phytoalexin from bean), have been engineered into transgenic plants using the *Agrobacterium*-mediated system. Increasing the levels of chitinase(s) by introducing foreign chitinase genes in transgenic plants has been proposed for a long time to be a possible way to enhance plant resistance against fungal pathogens (Nitzsche, 1983). Thus, a critical question that may be addressed is whether the overexpression of a cloned chitinase gene in transgenic plants can enhance resistance or tolerance of plants to infection by chitin-containing fungi (Punja and Zhang, 1993).

Successful introduction of cloned chitinase genes or their promoter sequences from different sources into plant species has been reported in several studies. Jones *et al.* (1988) studied the effects of using different promoters, from *rbcS* gene and from *cab* genes, on the expression of a

bacterial chitinase gene from *S marcescens* (*chiA*) that was transferred into tobacco by *Agrobacterium*-mediated transformation. The use of the *rbcS* promoter resulted in higher (ca. 3-fold) bacterial chitinase mRNA than when a *cab* promoter was used. Transgenic plants accumulated *chiA* proteins up to 0.25% of the total soluble leaf proteins and showed significantly higher chitinase activity than nontransformed plants. The transgenic plants expressing *S. marcescens* chitinase were tested for resistance using different fungi. Reduced disease symptoms were observed in a test with *Alternaria longipes* using a standardized leaf disc assay (Dunsmuir and Suslow, 1989) as well as in greenhouse and field trials with *Rhizoctonia solani* (Dunsmuir *et al.*, 1992).

Promoters of chitinase genes originating from different plants, including rice, *A. thaliana* and bean, were transferred into tobacco, and the activity of the promoter in the transgenic plants was studied by fusion with the *GUS* reporter gene (Broglie *et al.*, 1989; Roby *et al.*, 1990; Samac and Shah, 1991; Zhu *et al.*, 1993). It was shown from these studies that the promoters were induced by pathogen attack, ethylene and elicitors.

In a study by Linthorst *et al.* (1990), basic and acidic chitinase genes from TMV-infected tobacco and petunia, respectively, were transferred into tobacco. Transgenic tobacco containing the basic chitinase gene accumulated the enzyme intracellularly and the enzyme was targeted correctly to the central vacuole. The acidic chitinase was constitutively expressed and accumulated extracellularly. This study suggests that tobacco is able to recognize the vacuolar targeting signals of petunia chitinase. The expression levels of heterologous chitinase genes in transgenic plants

varied in different organs, and at different stages of plant development (Broglie *et al.*, 1991; Zhu *et al.*, 1993).

When transgenic plants expressing heterologous chitinase genes are challenged with chitin-containing fungal pathogens, the responses of the transgenic plants vary greatly. Broglie *et al.* (1991) produced hybrid gene constructs containing a fusion of CaMV *35S* constitutive promoter and a bean endochitinase *CH5B* gene, and transferred the constructs into tobacco and canola via *Agrobacterium*-mediated transformation. The transgenic plants exhibited a wide range of chitinase activity, but some displayed significant increases in the constitutive activity, as compared with control plants. The highest were 44-fold and 33-fold higher in leaves of homozygous progenies of transgenic tobacco and canola, respectively. Enhanced resistance was observed in the transgenic tobacco plants when they were tested in soil infested with *R. solani*. A detailed ultrastructural and cytochemical study on transgenic and nontransgenic canola infected by *R. solani* showed that chitin breakdown, fungal lysis and alteration of fungal growth were observed typically in the transgenic but not in the nontransgenic plants (Benhamou *et al.*, 1993). This suggested that the enhanced protection against attack by the pathogen might have resulted, at least in part, from the constitutive expression of the bean endochitinase gene.

Neuhaus *et al.* (1991b) transferred a fusion of 35S promoter and tobacco Class I (basic) chitinase gene into *Nicotiana sylvestris*. A chitin-containing fungus, *Cercospora nicotianae*, was used to infect the transgenic plants. The transgenic plants exhibited an extremely high accumulation of

chitinase (up to 120-fold) compared with that of control plants. However, the high levels of chitinase resulted in only a slight increase in resistance to *C. nicotianae.* This indicated that Class I chitinase might not be the limiting factor in the resistance against this specific fungus. Since *C. nicotianae* initially grows intercellularly, the intracellular localization of the chitinase in the transgenic plants might not have functioned early enough in response to this pathogen (Punja and Zhang, 1993). Some of the transgenic lines showed lower overall chitinase levels than the nontransgenic plants, which suggested that the transgene might have inhibited the expression of the homologous host gene (Neuhaus *et al.*, 1991b). In a similar study, a gene construct that contained a class-III acidic chitinase gene from sugar beet (SE2), regulated by an enhanced *35S* promoter and the *35S* terminator, was transferred into *Nicotiana benthamiana* (Nielsen *et al.*, 1993). In transgenic plants, the SE2 chitinase is excreted into and accumulated in the intercellular space of leaves. When transgenic lines which exhibited high extracellular levels of SE2 were evaluated for resistance against *C. nicotianae,* however, no increase in resistance was observed compared with that of control plants.

Several recent studies have focused on increasing the effectiveness of using heterologous chitinases in transgenic plants. Some vacuolar (basic) chitinases have been suggested to have a greater antifungal activity but are expected to act later during pathogen infection (Punja and Zhang, 1993). Vacuolar PR-proteins can be targeted extracellularly, by modification of their C-terminal propeptide (Melchers *et al.*, 1993), which opens the possibility of more effective use of basic vacuolar chitinase in transgenic

plants when challenged with fungal pathogens. In addition, class I chitinase has been shown to act synergistically with β-1,3-glucanase *in vitro* (Sela-Buurlage *et al.*, 1993). Although some fungi are susceptible to lysis by a basic chitinase, many other pathogenic ones, such as *Fusarium solani*, *Fusarium oxysporum* and *Alternaria solani* are affected only by a combination of chitinase and β-1,3-glucanase (Mauch *et al.*, 1988). Thus, research is being done to use a combination of chitinase and β-1,3-glucanase in transgenic plants, to improve their effectiveness. In one approach, a four-gene construct encoding chitinases (classes I and II) and glucanases (classes I and II), under control of a CaMV *35S* promoter, was developed and introduced into tomato (Van den Elzen *et al.*, 1993). Four of eight transgenic lines obtained showed significantly improved resistance when challenged with *F. oxysporium*. A negative correlation was observed between expression levels of the transgenes and disease severity in a fungal assay. It was suggested also that there was a synergistic action *in planta* between class I chitinase and class I glucanase used in this study (Van den Elzen *et al.*, 1993).

Using a different approach, Zhu *et al.* (1994) investigated protective interactions between an acidic chitinase and a basic glucanase by infection assays on the tobacco progenies from crosses between transgenic lines exhibiting strong constitutive expression of the respective foreign enzymes. Hybrid plants containing a rice basic (class I) chitinase (RCH10) gene and an alfalfa acidic chitinase (AGLU1) gene, each under the control of CaMV 35S enhancer and double promoter, respectively, were generated by crossing

Table 1.1. Genetically engineered plants with heterologous chitinase genes and responses of the transgenic plants when challenged with pathogenic fungi

Transgenic plants	Gene source	Class	Trans/Ctl. Ratio[a]	Pathogen Tested	Increase tolerance	Reference
tobacco	bean	I	≤44	*R. solani*	yes	Broglie *et al.*, 1991
				Pythium sp.	no	Broglie *et al.*, 1991
canola	bean	I	≤33	*R. solani*	yes	Broglie *et al.*, 1993
N.sylvestris	tobacco	I	19-20	*C. nicotianae*	no	Neuhaus *et al.*,1993
N.benthamina	sugarbeet	III	na	*C. nicotianae*	no	Nielsen *et al.*, 1993
tomato	tobacco	I	na	*F. oxysporium*	no[b]	Van den Elzen *et al.*, 1993
tobacco	rice	I	8	*C. nicotianae*	slighly[c]	Zhu *et al.*, 1994
tobacco	*S.marcescens*	exo	1.4	*A. longipes*	yes	Jones *et al.*, 1988; Dunsmuir
				R. solani	yes	and Suslow, 1989; and Dunsmuir *et al.*, 1992
tobacco	*S.marcescens*	exo	na	*R. solani*	yes	Howie *et al.*, 1994
				Pythium ultimum	no	

[a] Chitinase activity ratio of transgenic vs. control plants

[b] Significantly increased when a glucanase gene from tobacco was used with the chitinase gene

[c] Significantly increased when an acidic glucanase gene from alfalfa was used with the chitinase gene.

homozygous transgenic lines containing each of the transgenes. The transgenic lines used for crossing parents were confirmed to contain each of the transgenes in single loci. Results from infection assays using *C. nicotianae* showed that disease development was very strongly reduced (ca. 90%) in homozygous (2n/2n) and (ca. 40%) in heterozygous (1n/1n) plants containing both transgenes, and the reductions were greater than when homozygous (2n) transgenic plants containing either the chitinase or the glucanase transgenes were used separately.

1.8 Rationale and Objectives

Cucumber is an important vegetable crop and is commonly cultivated in many areas in the world, from temperate to tropical regions. Conventional breeding have developed many cultivars with various superior characteristics, including resistance to certain pests and diseases. Majority of the cultivars, however, are susceptible to several major diseases, such as powdery mildew, *Fusarium* wilt, root rot, anthracnose, and some virus diseases. Transfer of some desirable traits, such as disease resistance, from other species or genera in the Cucurbitaceae through conventional breeding has met with difficulties due to sexual incompatibility.

Genetic transformation using *A. tumefaciens* offers considerable potential for the introduction of disease resistance genes from different sources into cucumber. To explore this possibility, a reliable regeneration system from tissues cultured *in vitro* is required. The capacity of plantlet regeneration from cucumber cell and tissue cultures is dependent on

genotype as well as culture conditions. This necessitates determining the optimal growth conditions to achieve a high rate of regeneration, depending on the cultivars of cucumber that are deployed. Most studies on *Agrobacterium*-mediated transformation of this crop have dealt with the introduction of *NPT II* or *GUS* gene, and only one study has used an agronomically useful gene, i.e. the gene coding for CMV coat protein. Introduction of chitinase genes, which may result in the enhancement of plant resistance against diseases, would provide additional information on the potential use of a foreign, agronomically useful trait in transgenic cucumber.

The objectives of this research are:

1. To evaluate factors influencing plantlet regeneration in tissue culture of cucumber (*C. sativus*) and to develop a procedure for efficient plantlet regeneration from suspension cultures of cucumber cv. Endeavor.
2. To study the effects of various explant sources, and types and concentrations of plant growth regulators, on regeneration of *C. metuliferus* in tissue culture.
3. To introduce foreign chitinase genes into cucumber cv. Endeavor using *A. tumefaciens*.
4. To evaluate the transgenic plants containing chitinase transgenes for disease development when challenged with several pathogenic fungi.

CHAPTER II

EVALUATION OF FACTORS INFLUENCING PLANTLET REGENERATION IN TISSUE CULTURE OF CUCUMBER, *CUCUMIS SATIVUS*

2.1 Introduction

Plant tissue culture is a potential tool for the improvement of crop plants. In cucumber, the availability of dependable methods for plant regeneration from tissue culture is essential to take advantage of biolological alternative for genetic improvement, such as by *Agrobacterium*-mediated transformation (Trulson and Shahin, 1986; Sarmento *et al.*, 1992) or by protoplast fusion (Punja and Raharjo, 1993; Tang and Punja, 1989). Although whole plants have been regenerated from cultured cells and tissues of various cucumber cultivars, either via somatic embryogenesis or organogenesis (reviewed in Chapter I), there are still inherent problems in using tissue culture technique in cucumber. The most important are low regeneration capacity and abnormal development of plantlets. Response and regeneration capacity in cucumber tissue culture is dependent on genotype factor, explants souces, medium factor and balance of plant growth regulators (Novak and Dolezelova, 1982; Punja *et al.*, 1990b; Wehner *et al.*, 1990). This necessitates evaluation of the effects of those factors when a high regeneration capacity is to be achieved in certain cultivars.

In many plant species, addition of $AgNO_3$ to tissue culture media is useful to enhance response and plantlet regeneration from cultured tissues or calli (Purnhauser *et al*, 1987; Williams et al., 1990; Songstad *et al*, 1991; Roustan et al., 1992) as well as to increase regeneration frequency following *Agrobacterium*-mediated transformation (De Block, 1988; Marton and Browse, 1991; Mukhopadhyay *et al.*, 1992). Since the use of $AgNO_3$, which is known to function as an inhibitor of ethylene action (Beyer, 1976), might not have an effect or even inhibit regeneration from tissue culture in some other studies, it is suggested that the response to the presence of ethylene inhibitors such as $AgNO_3$ should be addressed with each species (Songstad *et al.*, 1991). No reports have been published concerning the use of $AgNO_3$ in tissue culture of cucumber.

Agrobacterium-mediated transformation generally involves the use of dominant selectable markers which can express in transformed cells of tissues and transgenic plants. Among the most commonly used selectable marker is resistance to kanamycin, which is conferred by the *NPT II* gene (Walden, 1987). Since the level of tolerance to kanamycin varies from one plant species to another and even among different explants of the same species (Mathews, 1988), it is important to determine the kanamycin tolerance level of various tissues of a plant species to achieve efficient selection.

The focus of this study was to evaluate various factors that might affect the frequency of plantlet regeneration in cucumber tissue cultures and to determine the optimal conditions which could be applicable for subsequent use in *Agrobacterium*-mediated transformation. This study

consists of three parts. The objective of the first part was to evaluate the effects of cultivar, explant source, combinations of auxin and cytokinin, and incubation conditions, on plantlet regeneration of cucumber. The second part was to test the effect of addition of $AgNO_3$ in the culture medium on regeneration frequencies from tissue culture of cucumber cv. Endeavor. The last part of this study was to test the effect of kanamycin concentration on growth and regeneration capacity in cucumber tissue culture.

2.2 Materials and Methods

2.2.1 Plant materials

Three cucumber cultivars were used; two were pickling cultivars, Endeavor and Calypso (both F_1 hybrids, seeds provided by Campbell Soup Co., Davis, CA) and the third was a long English cultivar, Fidelio (an F_1 hybrid, seeds provided by De Ruiter Seeds Inc., OH). For preparation of sterile seedlings as explant sources, seeds were washed with detergent and rinsed in running cold tap water and their seed coats were removed using a pointed forcep. This was followed by surface-sterilization in 70% ethanol for 30 s, a 4-min soak in a 10% solution of commercial bleach (Javex, 6.25% sodium hypochlorite) and three rinses in sterile distilled water. Seedlings were grown in Magenta GA-7 culture vessels (Magenta Corp., Chicago, IL) containing 50 ml of half-strength MS basal medium (Murashige and Skoog, 1962) (one-half macro- and micro-elements, 100 $mg \cdot l^{-1}$ myo-inositol, 0.1 $mg \cdot l^{-1}$ thiamine-HCl, 0.5 $mg \cdot l^{-1}$ nicotinic acid, 0.5 $mg \cdot l^{-1}$ pyridoxine-HCl,

2 $mg \cdot l^{-1}$ glycine, 30 $g \cdot l^{-1}$ sucrose, 10 $g \cdot l^{-1}$ agar). All chemicals and agar used were tissue-culture grade (Sigma Chemical Co., St. Louis, MO). The pH of the medium was adjusted to 5.8 prior to addition of the agar and the medium was autoclaved (at 121°C, ≈1.05 $kg \cdot cm^{-2}$, for 20 min). Stock solution (200x) of mixtures of thiamine-HCl, nicotinic acid, pyridoxine-HCl, and glycine was filter-sterilized (0.2-μm pores) and added to the medium after autoclaving. Ampicillin (filter-sterilized, 0.2-μm pores) was added to a final concentration of 100 $g \cdot l^{-1}$ to reduce potential bacterial contamination. To ensure uniform germination, the culture vessels with seeds were incubated in the dark at 29°C for 2 d prior to placing them in the light (provided by cool-white fluorescent lamps, ≈30 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at culture level) at ambient temperatures of 25-29°C with a photoperiod of 16 $h \cdot d^{-1}$. Sterile seedlings were used as explants sources in all experiments.

2.2.2 Tissue Culture Medium

The medium used in all experiments was full-strength MS basal medium (macro- and micro-elements, vitamins and sucrose), with 100 $mg \cdot l^{-1}$ ampicillin and gelled by addition of 10 $g \cdot l^{-1}$ agar. The pH of the medium was adjusted to 5.8. The medium was sterilized by autoclaving (at 121°C, ≈1.05 $kg \cdot cm^{-2}$, for 20 min). Approximately 25 ml of medium was dispensed into disposable Petri dishes (20x100 mm). The types and levels of plant growth regulators added to the medium were according to treatment requirement.

2.2.3 Part 1: Effects of Cultivar, Explant Source, Combinations of Auxin and Cytokinin and Incubation Conditions on Plantlet Regeneration of Cucumber

Experiment 1. The cucumber cultivar used in this experiment was Endeavor. Explants used were petiole segments (4 mm in length) from young first and second leaves of 10- to 21-d-old seedlings. Depending on the treatment, combinations of plant growth regulators supplemented to the MS medium were 5.0 μM 2,4-D or 5.0 μM NAA combined with 5.0 μM BA or 5.0 μM kinetin. All plant growth regulators were added before the medium was autoclaved.

Nine explants were placed in each Petri dish which was then sealed with Parafilm®. The number of replicate dishes per treatment was 9 to 10. Following placement of the explants onto the medium, the culture dishes were incubated under light (provided by cool-white fluorescent lamps, intensity of 45 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at culture level) at a photoperiod of 16 $h \cdot d^{-1}$, at ambient temperatures of 25-29°C. Data on callus growth, root formation, and regeneration of somatic embryos and shoots were recorded 6-7 weeks after placement of the explants on the medium.

Experiment 2. Cucumber cultivars tested in this experiment were Endeavor, Calypso and Fidelio. Three types of explants were used: a) cotyledons (5x5 mm^2 in size, from 5-d-old seedlings), b) leaf segments (5x5 mm^2 segments, from young first and second leaves of 10- to 21-d-old seedlings), and c) petioles (4 mm^2 segments, from young first and second leaves of 10- to 21-d-old seedlings). Cotyledon and leaf explants were placed

on the medium adaxial side up and petiole explants were placed horizontally on the medium.

Depending on the treatment, combinations of plant growth regulators supplemented to the MS medium included: a) NAA/zeatin (at 5.0/5.0 μM), b) NAA/BA (at 5.0/2.5 μM), or 2,4-D/BA (at 5.0/5.0 μM). All plant growth regulators used, except for zeatin, were added to the medium prior to autoclaving at 1.05 $kg \cdot cm^2$ for 20 min; zeatin was filter-sterilized and added after autoclaving.

Six explants were placed in each Petri dish and the number of replicate dishes used for each treatment tested was 10. Following placement of the explants onto the medium, the culture dishes were incubated either directly under light conditions (provided by cool-white fluorescent lamps at an intensity of 45 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at culture level) at a photoperiod of 16 $h \cdot d^{-1}$ or in continuous darkness for the first 3 weeks before transfer to the light at the same intensity, all at ambient temperatures of 25-29°C. Data on callus growth, root formation, and regeneration of somatic embryos and shoots was recorded 6-8 weeks after placement of the explants on the medium.

2.2.4 Part 2: Effect of $AgNO_3$ on Tissue Culture of Cucumber cv. Endeavor

The effect of adding $AgNO_3$ to the medium (at a concentration of 20 μM), on callus performance and plantlet regeneration from cucumber tissue culture, was evaluated. The explant type used was petiole segments, 4

mm in length, from young first and second leaves of 10- to 21-d-old seedlings. Plant growth regulators added to the MS medium were 2,4-D and BA each at 2.5/2.5, 5.0/5.0 or 7.5/7.5 μM. The medium was either with or without the addition of 20 μM $AgNO_3$. Filter-sterilized stock solution of $AgNO_3$ was added to the final concentration after sterilization of the medium.

Ten explants were placed in each Petri dish and the number of replicate dishes per treatment was five. Following placement of the explants onto the medium, the culture dishes were incubated in continuous darkness for 3 weeks, followed by transfer to the light (provided by cool-white fluorescent lamps at an intensity of 45 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at culture level) at a photoperiod of 16 $h \cdot d^{-1}$, and ambient temperatures of 25-29°C. Data on callus growth and appearance, and regeneration of somatic embryos and shoots were recorded at week 6 to 7 after placing explants on the medium.

2.2.5 Part 3: Effects of Kanamycin on Regeneration from Tissue Culture of Cucumber

To determine the optimal kanamycin concentrations in selective media for use in *Agrobacterium*-mediated transformation studies, various concentrations were tested. The explant type used was petiole segments, 4 mm in length, from young first and second leaves of 10- to 21-d-old seedlings. Tissue culture medium used was full-strength MS supplemented with 2,4-D/BA (5.0/5.0 μM). Sterile stock solution of kanamycin (filter-sterilized) was added to the medium after sterilization when the medium

was still warm. Various concentrations of kanamycin, i.e. 0, 10, 20, 40, 60, 80 and 100 $mg \cdot l^{-1}$, were tested.

Twelve petiole explants were placed in each Petri dish and the number of replicate dishes per treatment was six. Following placement of the explants onto the medium, the culture dishes were incubated in darkness for 4 weeks followed by transfer to the light (provided by cool-white fluorescent lamps, intensity of 45 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at culture level) at a photoperiod of 16 $h \cdot d^{-1}$, and ambient temperatures of 25-29°C.

2.3 Results

2.3.1 Effects of Cultivar, Explant Source, Combinations of Auxin and Cytokinin and Incubation Conditions

Various explant types used in this study underwent regeneration of plantlets, generally through initiation and growth of calli from initial explants. This callus development was followed either by formation of embryogenic (yellow and friable) sectors of calli and development of somatic embryos (in somatic embryogenesis) (Fig. 2.1 a and b), or by development of shoot buds (in organogenesis) (Fig. 2.1 c).

One week after placement onto culture medium, explants swelled and started to form calli. Most of the explants began to form calli during the first and second week of experiment in most treatments tested, except those using leaf explants with the dark pre-treatment (in Experiment 2) in which callus formation was less and began later (during the second or third week

of experiment). The frequencies of callus formation were generally high regardless of the treatment (Tables 2.1 and 2.2). The texture, colour, and size of the calli differed, however, depending on the plant growth regulator used, especially the auxin, and the light/dark pre-treatment.

Somatic proembryo formation, typically occurring on medium with 2,4-D, was initiated by development of yellowish-white granular structures on the surface of calli or callusing explant tissues. The structures subsequently developed into somatic embryos which appeared as small, hyaline protuberances and finally enlarged into torpedo-shaped embryos. During their development, the appearance and color changed from hyaline and pale yellow to opaque and green. In some cases, somatic embryos were formed directly on greenish and compact calli, when a dark-pretreatment was not given. Roots developed from calli or initial explant tissue, with frequencies of their formation depending on treatment. In Experiment 1, which employed different combinations of auxin (2,4-D or NAA) and cytokinin (BA or kinetin), the greatest percentage of organ formation (via somatic embryos and shoot bud formation) was achieved when a combination of NAA and BA was used in the medium, followed by a combination of 2,4-D and BA (Table 2.1). A combination of either type of auxin and kinetin tended to induce a greater percentage of root formation from the calli, i.e. 91.4% in NAA/kinetin medium and 42.2% in 2,4-D/kinetin medium. This occurred at the expense of the capacity for regeneration of somatic embryos and shoots, so that regeneration frequencies were very low in the medium with kinetin.

All factors tested in Experiment 2, i.e. explant type, plant growth regulator regime and culture pre-treatment, significantly influenced the regeneration capacity ($P<0.01$) in the three cultivars used, except for dark culture pre-treatment in cultivar Fidelio. However, the effect of the interactions among the three factors tested on the regeneration frequency was also significant (Table 2.3). Among the three explant types, petiole segment was the explant type which generally resulted in the highest regeneration capacity, except in cultivar Fidelio on 2,4-D/BA medium. In most cases, 3-week dark pre-treatment increase percentage of calli with regeneration of somatic embryos or shoots. Dark-pretreated explants usually produced calli which appeared paler (more whitish-yellow) and less compact than their counterparts incubated in the light. The effects of plant growth regulator regime were shown to be dependent on explant type used. For instance, with cotyledon explants, the greatest regeneration frequency was achieved on NAA/BA medium; with leaf explant, this was achieved on NAA/Z medium. Among all treatments in Experiment 2, however, the greatest regeneration percentage (80.0%) was achieved using petiole explants on 2,4-D/BA medium with dark pre-treatment, followed by NAA/BA medium (also with dark pre-treatment; 76.7%). Although Experiment 2 was not designed to compare the capacity of somatic embryo or shoot regeneration of the three cultivars used, there was a difference in regeneration capacity among the three cultivars (Table 2.3). The average regeneration frequencies in cultivars Endeavor, Calypso and Fidelio were 31.5%, 13.1% and 4.3%, respectively.

2.3.2 Effect of $AgNO_3$

Nearly all of the explants in all treatments in the test using $AgNO_3$ formed calli. However, the differences in the percentage of callus production among treatments was not statistically significant ($P \geq 0.05$). There were significant effects of the various combinations of 2,4-D/BA or the use $AgNO_3$ on the regeneration frequency in cultivar Endeavor (Fig 2.2). However, the effects of the interaction between the concentration of 2,4-D/BA and the use of $AgNO_3$ was not significant. Based on visual observation, calli on medium with $AgNO_3$ appeared to be more compact than those without $AgNO_3$. Upon transfer from the dark to incubation with light (16-h$\cdot d^{-1}$ photoperiod), the pale yellow compact calli turned green and became very compact, but they developed somatic embryos or shoots less frequently did than those on medium without $AgNO_3$.

2.3.3 Effect of Kanamycin

An increase in kanamycin concentration from 0 to 40 mg$\cdot l^{-1}$ resulted in a reduction in callus growth and formation of somatic embryos (Table 2.4), with a drastic reduction in regeneration frequency from 69.2% to 13.9% when the kanamycin concentration was increased from 10 to 20 mg$\cdot l^{-1}$. At a concentration of 40 mg$\cdot l^{-1}$ or higher, explants barely grew and no embryo formation occurred.

Table 2.1. Percentage of callus formation, root formation and regeneration of plantlets from tissue culture of cucumber cv. Endeavor as influenced by different auxin/cytokinin combinations.

Plant Growth Regulator Combination	Callus Formation (%)	Root Formation (%)	Regeneration (%)
2,4-D/BA (5.0/5.0 μM)	98.9	3.3 a	45.6 b
2,4-D/kin (5.0/5.0 μM)	98.9	42.2 b	16.7 a
NAA/BA (5.0/5.0 μM)	100.0	14.8 ab	49.4 b
NAA/kin (5.0/5.0 μM)	100.0	91.4 c	2.5 a
Tukey's HSD (0.05)	-	27.7	26.4
Significance	ns	**	**

- Each value represents mean of 10 replications, each with 9 explants, per treatment, rated in week 6-7. Values in the same coloumn which are followed by the same letter are not significantly different (Tukey's HSD, P=0.05).
- ns, analysis of variance showing no significance ($P \geq 0.05$).
- **, analysis of variance showing significance ($P < 0.01$).

Table 2.2. Influence of explant source, plant growth regulator combinations and light/dark culture pre-treatment on callus formation in cucumber cv. Endeavor, Calypso and Fedelio

Explant Type	Plant Growth Regulators Combinations	Culture Pre-treatment	Percentage of Explants Forming Calli [a)]		
			Endeavor	Calypso	Fidelio
Cotyledon	NAA/Z (5.0/5.0 µM)	light	100	100	100
		dark	100	100	100
	NAA/BA (5.0/2.5 µM)	light	100	95.0	100
		dark	100	100	100
	2,4-D/BA (5.0/5.0 µM)	light	100	96.7	100
		dark	100	100	100
Leaf	NAA/Z (5.0/5.0 µM)	light	88.3	93.3	90.0
		dark	61.7	55.0	45.0
	NAA/BA (5.0/2.5 µM)	light	98.3	85.0	73.3
		dark	46.7	38.3	40.0
	2,4-D/BA (5.0/5.0 µM)	light	73.3	60.0	66.7
		dark	20.0	33.3	21.7
Petiole	NAA/Z (5.0/5.0 µM)	light	100	100	95.0
		dark	100	98.3	100
	NAA/BA (5.0/2.5 µM)	light	100	100	98.3
		dark	100	100	98.3
	2,4-D/BA (5.0/5.0 µM)	light	95.0	96.7	100
		dark	100	100	96.7

Each value represents mean of 10 replications, each with 6 explants, per treatment, rated in week 4-5.

Table 2.3. Influence of explant source, plant growth regulator combination and light/dark culture pre-treatment on the regeneration of cucumber cv. Endeavor, Calypso and Fedelio

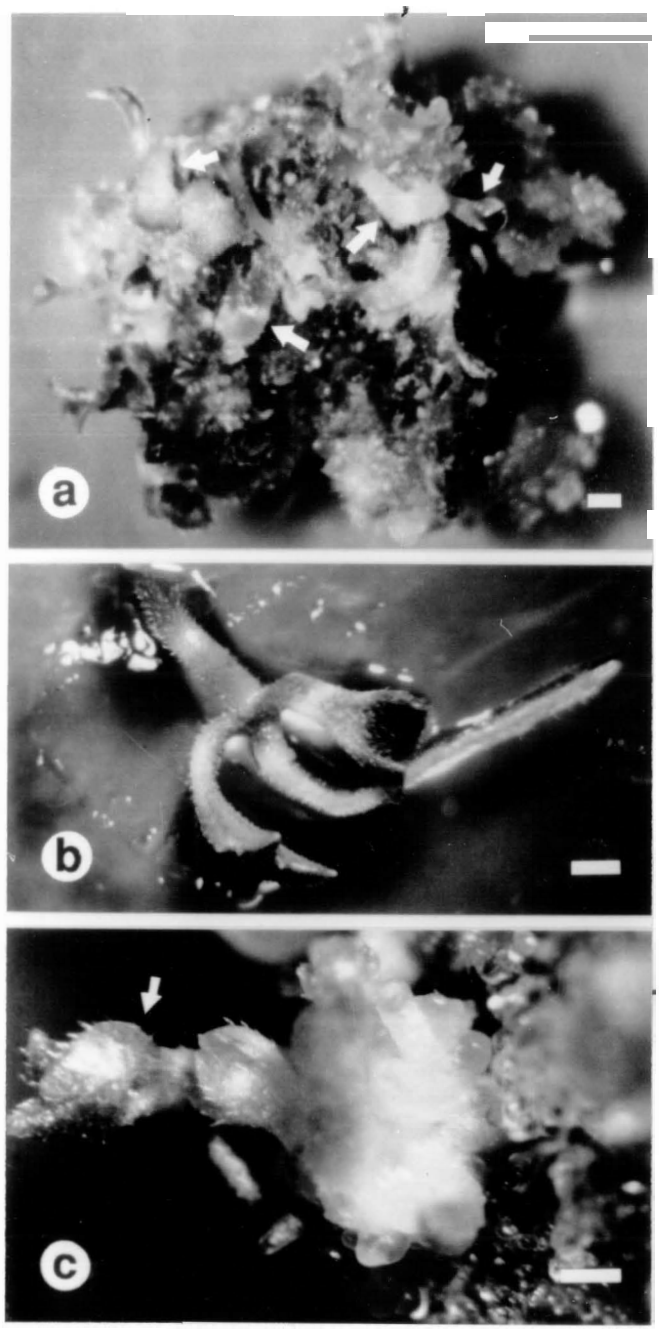
Explants	Plant Growth Regulators	Culture Pre-treatment	Percentage of Calli with Regeneration of Embryos or Shoots [a]		
			Endeavor	Calypso	Fidelio
Cotyledon	NAA/Z (5.0/5.0 µM)	light	11.7	3.3	0.0
		dark	28.3	0.0	0.0
	NAA/BA (5.0/2.5 µM)	light	40.0	8.3	0.0
		dark	15.5	18.3	0.0
	2,4-D/BA (5.0/5.0 µM)	light	5.0	23.3	0.0
		dark	1.7	31.6	0.0
Leaf	NAA/Z (5.0/5.0 µM)	light	21.7	3.3	0.0
		dark	41.7	5.0	0.0
	NAA/BA (5.0/2.5 µM)	light	8.3	8.3	3.3
		dark	6.7	8.3	0.0
	2,4-D/BA (5.0/5.0 µM)	light	3.3	0.0	5.0
		dark	0.0	3.3	0.0
Petiole	NAA/Z (5.0/5.0 µM)	light	58.3	11.7	5.0
		dark	70.0	38.3	5.0
	NAA/BA (5.0/2.5 µM)	light	51.7	28.3	3.3
		dark	76.7	38.3	8.3
	2,4-D/BA (5.0/5.0 µM)	light	45.0	1.7	20.0
		dark	80.0	3.3	26.6
Average			31.5	13.1	4.3
Tukey's HSD (0.05)			10.1	8.3	6.5
Significance:	explant (E)		** [b]	**	**
	plant growth regulator (P)		**	**	**
	culture pre-treatment (C)		**	**	ns
	ExP		**	**	**
	ExC		**	**	**
	CxD		**	ns [c]	ns
	ExPxC		**	**	**

[a] Each value represents mean of 10 replications, each with 6 explants, per treatment, rated in week 8.

[b] **, analysis of variance showing significance ($P<0.01$).

[c] ns, analysis of variance showing no significance ($P\geq0.05$).

Fig 2.1. Morphogenesis in cucumber tissue on MS medium with different plant growth regulators. **a)** Somatic embryos formed on the surface of a callus developing from a petiole explant, typically occurring on medium with 2,4-D and BA (*arrows*). **b)** Development of a somatic embryo, characterized by growth of shoot and root polars, into a small plantlet after transfer onto medium lacking of plant growth regulator. **c)** Shoot formation (*arrow*) from a callus (organogenesis, no root polar), typically occurring on medium with NAA and BA. Bar = 1 mm.


a
b
c

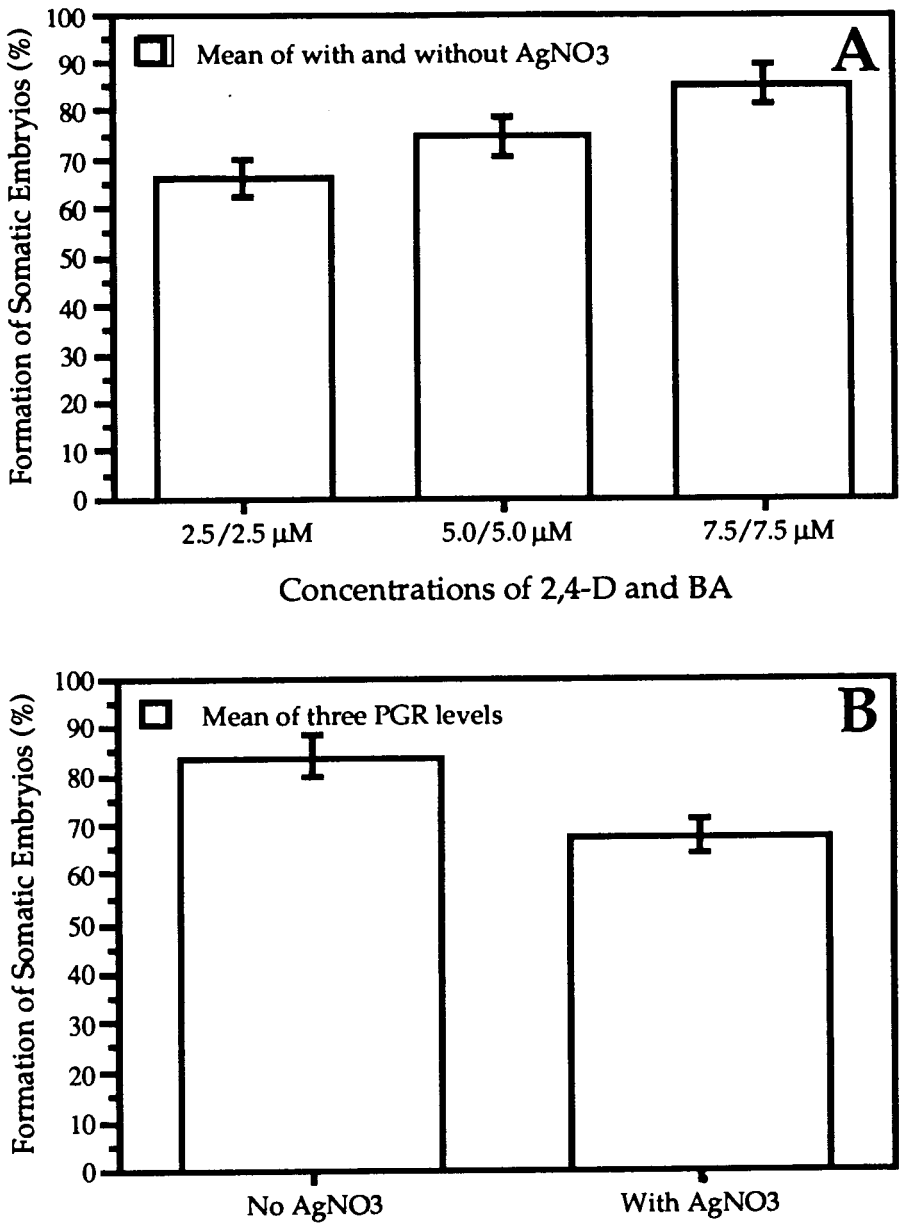


Figure 2.2. Percentage of regeneration of somatic embryos from tissue culture of cucumber cv. Endeavor; **a)** at different concentrations of 2,4-D/BA, **b)** with or without addition of $AgNO_3$. Bars represent Tukey's HSD at P=0.05

Table 2.4. Effects of kanamycin concentration on callus growth and embryo regeneration of cucumber cv. Endeavor

Kanamycin Concentration ($mg.L^{-1}$)	Diameter of Explants + Calli (mm)	Regeneration Rate (%)	Number of Somatic Embryos per Responding Explants
0	10.7 ± 0.7 [a)]	71.9 ± 7.8	7.7 ± 1.2
10	8.6 ± 0.4	69.2 ± 8.3	6.9 ± 1.4
20	7.7 ± 0.4	13.9 ± 5.1	2.4 ± 0.9
40	5.7 ± 0.3	0.0	-
60	5.5 ± 0.2	0.0	-
80	4.8 ± 0.1	0.0	-
100	5.0 ± 0.1	0.0	-

a) Each value represents mean ± standard error (SE)

2.4 Discussion

The results from this study showed that there was no difference in the percentage of callus formation when explants were incubated on medium with different plant growth regulator regimes, when petiole or cotyledon explants are used. However, the capacity for regenerating somatic embryos or shoots was clearly dependent upon plant growth regulator regime. The ability of cucumber tissue cultures to undergo somatic embryogenesis or organogenesis in certain culture conditions was not reflected by the ability of the explants to form calli in this study, which is similar to what has been shown in other studies. In several instances, calli can be induced easily using various combinations of an auxin and a cytokinin, but regeneration of somatic embryos or shoots may only occur at a low frequency or not at all (Kim *et al.*, 1988; Punja *et al.*, 1990b).

In this study, the use of petiole explants gave the highest frequency of regeneration in all three cultivars used. This is consistent with a previous study with pickling cucumber (Punja *et al.*, 1990b), in which petiole segments were used as explants. However, in this study dark pre-treatment did not consistently have a positive effect on regeneration in all treatments, except when petiole explants were used.

The greatest regeneration capacity, in terms of percentage of explants forming somatic embryos and/or shoots, was achieved on NAA/BA at 5.0/5.0 μM (49.4%), which was not statistically different from that achieved on 2,4-D/BA at 5.0/5.0 μM (45.6%), the dominant type of regeneration occurring in those two treatments differed. In the medium containing

NAA (in both experiment 1 and 2), regeneration was dominated by shoot bud formation (organogenesis) from friable calli, with some somatic embryos also developing on the calli. On the medium with 2,4-D, calli which were formed were usually smaller and more compact, and regeneration occurred exclusively by formation of somatic embryos. This is consistent with previous reports which indicated organogenesis on culture media containing NAA (Wehner and Locy, 1981; Kim *et al.*, 1988; Cade *at al.*, 1990a) and somatic embryogenesis on media with 2,4-D (Bergervoet and Custer, 1986; Trulson and Shahin, 1986; Cade *et at.*, 1990b; Punja *et al.*, 1990b).

$AgNO_3$ has been shown to improve the response and regeneration capacity in tissue cultures of many plant species (De Block, 1988; Marton and Browse, 1991; Songstad, 1991), in addition to *C. melo*, a close relative of cucumber (Roustan *et al.*, 1992). It was suggested that in *C. melo* ethylene inhibited regeneration via organogenesis, and addition of an inhibitor of ethylene action might be used to enhance regeneration efficiency. However, within the range of 2,4-D and BA concentrations used in this study (each at 2.5 to 7.5 μM), the addition of $AgNO_3$ in the culture medium did not have a positive effect on regeneration capacity in cucumber. This is probably because the action of ethylene, or disruption of auxin transport by ethylene (Beyer, 1976), was not the limiting factor for cucumber regeneration via somatic embryogenesis. In this study, however, $AgNO_3$ influenced the appearance and growth pattern of calli, whose underlying mechanism was unknown. In the presence of $AgNO_3$, calli appeared to be more compact, opaque, with more protuberance structures, and appeared

greener under the light conditions. However, this type of calli produced somatic embryos less frequently.

In *Agrobacterium*-mediated transformation of cucumber, kanamycin has been added to a selective medium at concentrations ranging from 25 $mg \cdot l^{-1}$ (Trulson *et al.*, 1986) to 200 $mg \cdot l^{-1}$ (Chee, 1990b). The levels of tolerance of plant tissues to selective agents, such as kanamycin, may vary not only in different plant species but also in different explants from the same species. In *B. juncea*, for instance, shoot regeneration from cotyledon explants was completely inhibited by an addition of kanamycin at as low as 20 $mg \cdot l^{-1}$ and shoot multiplication from shoot explants was hindered at 100 $mg \cdot l^{-1}$. In *Vigna radiata*, plant regeneration from cotyledon explants decreased at 100 $mg \cdot l^{-1}$ kanamycin or higher (Mathews, 1988). In this study, using petiole explants in a pickling cucumber cultivar, kanamycin at 20 $mg \cdot l^{-1}$ reduced regeneration frequency drastically and at 40 $mg \cdot l^{-1}$ or higher, no somatic embryo formation was obtained. Thus, using the selective medium with kanamycin at a concentration of 40 $mg \cdot l^{-1}$, the chance of selection escapes was reduced drastically.

CHAPTER III

PLANTLET REGENERATION FROM PETIOLE EXPLANTS OF THE AFRICAN HORNED CUCUMBER, *CUCUMIS METULIFERUS*

3.1 Introduction

C. sativus and *C. melo* are among members of the genus *Cucumis* which are the most popular and extensively cultivated (Robinson and Whitaker, 1974; Esquinas-Alcazar and Gilick, 1983; Singh, 1990). Most studies on tissue culture of *Cucumis* have, therefore, focused on establishing procedures for regeneration of cultivars within those two species (Kim *et al.*, 1988; Punja *et al.*, 1990b; Wehner *et al.*, 1990; Chee, 1991a). However, several other less extensively cultivated or wild *Cucumis* species have attracted interest because they possess useful traits not found in either *C. sativus* or *C. melo*. For example, *C. sativus* L. var. *hardwickii*, *C. metuliferus* Naud., and *C. anguria* L. are known to have increased vigor and fruit bearing capacity (Staub and Kupper, 1985), resistance to certain virus diseases and root knot nematodes (Fassuliotis, 1967; Provvidenti and Robinson, 1974; Punja *et al.*, 1988), and resistance to insects and nematodes (Esquinas-Alcazar and Gilick, 1983). In addition, *C. metuliferus* fruits are cultivated for ornamental purposes (Benzioni *et al.*, 1991). Regeneration of plantlets in *C. sativus* var. *hardwickii* and *C. anguria* from tissue explants has been accomplished (Orczyk *et al.*, 1988; Punja *et al.*, 1990a). To date,

however, attempts to achieve regeneration in the African horned cucumber, *C. metuliferus*, from tissue explants or protoplasts have been unsuccessful (Orczyk *et al.*, 1988; McCarthy *et al.*, 1989; Tang and Punja, 1989; Punja *et al.*, 1990a; Dabauza *et al.*, 1991). Explants of this species readily develop prolific callus (Orczyk *et al.*, 1988; Punja *et al.*, 1990a), with occasional root development (Orczyk *et al.*, 1988), on appropriate tissue culture media with cytokinin and auxin. Differentiation to form shoots may occur at a low frequency, depending on the plant growth regulator regimes (Punja *et al.*, 1990) but to date, plantlets have not been recovered. A procedure for plantlet regeneration from callused explants of *C. metuliferus* would be useful for propagation of the species, for its applicability to regeneration from callus derived from protoplasts (McCarthy *et al.*, 1989; Dabauza *et al.*, 1991), or in somatic hybridization studies to introgress some of the useful traits from *C. metuliferus* into *C. sativus* (Tang and Punja, 1989; Punja *et al.*, 1990a; Dabauza *et al.*, 1991; Punja and Raharjo, 1992).

The objective of this study was to evaluate the influence of various explant sources and growth regulator regimes and concentrations to achieve regeneration from explants of *C. metuliferus*.

3.2 Material and Methods

3.2.1 Plant materials

Seeds of *C. metuliferus* PI 292190 (courtesy of T.C. Wehner, North Carolina State University, Raleigh, NC) were used for the source of sterile

plant material. The seeds were surface-sterilized by dipping in 70% ethanol for 30 s, followed by soaking in a 10% solution of commercial bleach (Javex, 6.25% sodium hypochlorite) for 5 min and rinsing three times in sterile distilled water. To ensure uniform germination, the seeds were placed in Petri dishes containing sterile water and incubated in the dark at 29°C for 96 h. When the radicles had emerged, the seeds were transferred to Magenta GA-7 culture vessels (Magenta Corp., Chicago, IL) containing 50 ml of sterile half-strength MS basal medium with 100 $g \cdot l^{-1}$ ampicillin, pH 5.8. The culture vessels were incubated at ambient temperatures of 25-28°C with a photoperiod of 16 $h \cdot d^{-1}$ provided by cool-white fluorescent lamps. These *in vitro*-grown seedlings were used to provide explant sources in this study. The explants tested were: cotyledon segments (2x3 mm^2) from 10- to 14-d-old seedlings; leaf segments (3x3 mm^2) from the interveinal area of the second to third primary leaves; and hypocotyl and petiole segments (3 mm in length) from 3- to 4-week-old seedlings.

3.2.2 Medium and culture conditions

The medium used in all regeneration studies was full-strength MS medium containing vitamins (thiamine, pyridoxine, nicotinic acid), glycine, sucrose, agar and ampicillin at the concentrations described at Chapter II, subsection 2.2.1. The influence of auxins (2,4-D, NAA, IAA) and cytokinins (BA, kinetin, zeatin) was evaluated in various experiments as described below. All plant growth regulators were added to the medium before pH adjustment and autoclaving, except for zeatin which was filter-sterilized and added after autoclaving. Approximately 25 ml of medium

was dispensed into disposable Petri dishes (20x100 mm). Eight to twelve explants were placed in each Petri dish, which was sealed with Parafilm®. The number of replicate dishes used for each combination of plant growth regulators tested ranged from six to twelve. All dishes were incubated on culture racks under cool-white fluorescent lamps (intensity of 45 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at culture level), 16-$h \cdot d^{-1}$ photoperiod, and at ambient temperatures of 25-29°C.

3.2.3 Influence of growth regulator combinations

Combinations of auxin (IAA, NAA, 2,4-D; each at 1.0 μM) and cytokinin (BA, zeatin, kinetin; each at 1.0 μM) were used for callus initiation. Petri dishes with explants were incubated either directly in the light or placed in the dark for 2 weeks prior to transferring to the light. After 4 weeks, developing calli from the explants that were showing evidence of differentiation (bud or shoot-like structures) were transferred to MS medium containing either of BA, kinetin, or zeatin at 2.0 μM.

Combinations of NAA (2.0 μM) and BA (1.0 μM) , or 2,4-D (2.0 μM) and BA (1.0 μM) were used for callus initiation. After 4 weeks in the light, developing calli were either transferred to shoot initiation medium containing 4.0 μM zeatin, or were left on the callusing medium.

Combinations of 2,4-D (at 0.5, 1.0, 2.0, or 4.0 μM) and BA (1.0 μM), and combinations of NAA (at 0.5 or 2.0 μM) and BA (1.0 μM) were used for callus initiation. After 5 weeks, all calli were transferred to shoot initiation medium containing 2.0 μM zeatin.

Percentage of explants that formed callus, appearance of the calli, and evidence of differentiation were recorded 4-5 weeks after the experiments were initiated. Subsequently, calli were transferred to shoot initiation medium containing cytokinins, and the percentage of calli that formed shoots was recorded after 2-3 weeks. Plants were recovered by transfer of shoot-bearing calli to MS medium without growth regulators. Each experiment was repeated at least twice, and the data presented are the averages of these experiments.

3.3 Results

3.3.1 Callus formation and differentiation

All of the explant sources tested in this study (cotyledon, leaf, petiole, hypocotyl) developed callus to varying degrees on the various growth regulator combinations tested (Table 3.1). Data for the appearance of calli from cotyledon, leaf, or hypocotyl explants are not shown since petiole explants were the only tissues in which differentiation was ultimately achieved. The general appearance of the calli from petiole explants which were initially placed in either the light or dark for 2 weeks was similar (Table 3.1), except on media containing IAA. The typical friable, yellow callus which was observed on most growth regulator combinations is shown in Fig. 3.1 a. The only growth regulator combination in which the calli derived from petioles subsequently differentiated to form shoot-like structures was 2,4-D/BA at 1.0/1.0 μM (Fig. 3.1 b). Upon transfer of these

calli to MS medium which contained either BA, kinetin, or zeatin at 2.0 μM, further shoot development and proliferation occurred only on medium which contained zeatin (Fig. 3.1 c)

3.3.2 Regeneration

Callus, which was initiated from petiole explants on MS medium containing either of NAA/BA or 2,4-D/BA at 2.0/1.0 μM, showed evidence of differentiation into shoot-like structures after subculture onto medium containing zeatin (Table 3.2). In comparison, calli which were not subcultured did not differentiate. A range of concentrations of 2,4-D/BA and NAA/BA in the callus initiation medium was evaluated subsequently (Table 3.3). The optimal concentration was found to be 2.0/1.0 μM of 2,4-D/BA, which yielded differentiated calli at a frequency of about 21%. Upon subculture onto MS medium with 2.0 μM zeatin, shoot formation was obtained at a frequency of 14.6% (Table 3.3).

3.3.3 Plantlet development

From shoot proliferation medium containing zeatin, the shoots were excised from callused tissue after 2-3 weeks and transferred to Magenta vessels containing MS medium without plant growth regulators. The shoots elongated and developed roots at a frequency of about 65%. After 4-5 weeks on MS medium, they were transferred to sterilized natural soil in plastic pots. The plantlets were maintained in a growth chamber at 27°C, 16-h•d^{-1} photoperiod, and relative humidity (RH) of 85%, where they developed into plants of normal appearance.

Figure 3.1. Callus growth and differentiation from petiole explants of *Cucumis metuliferus* on MS medium. **a)** Prolific callus growth on medium containing NAA/BA at 1.0/1.0 μM. **b)** Differentiation of callus on medium containing 2,4-D/BA at 1.0/10 μM. **c)** Shoot development from differentiated callus initiated on medium with 2,4-D/BA at 1.0/1.0 μM and subcultured onto medium containing zeatin at 2.0 μM. Bar = 1 mm.

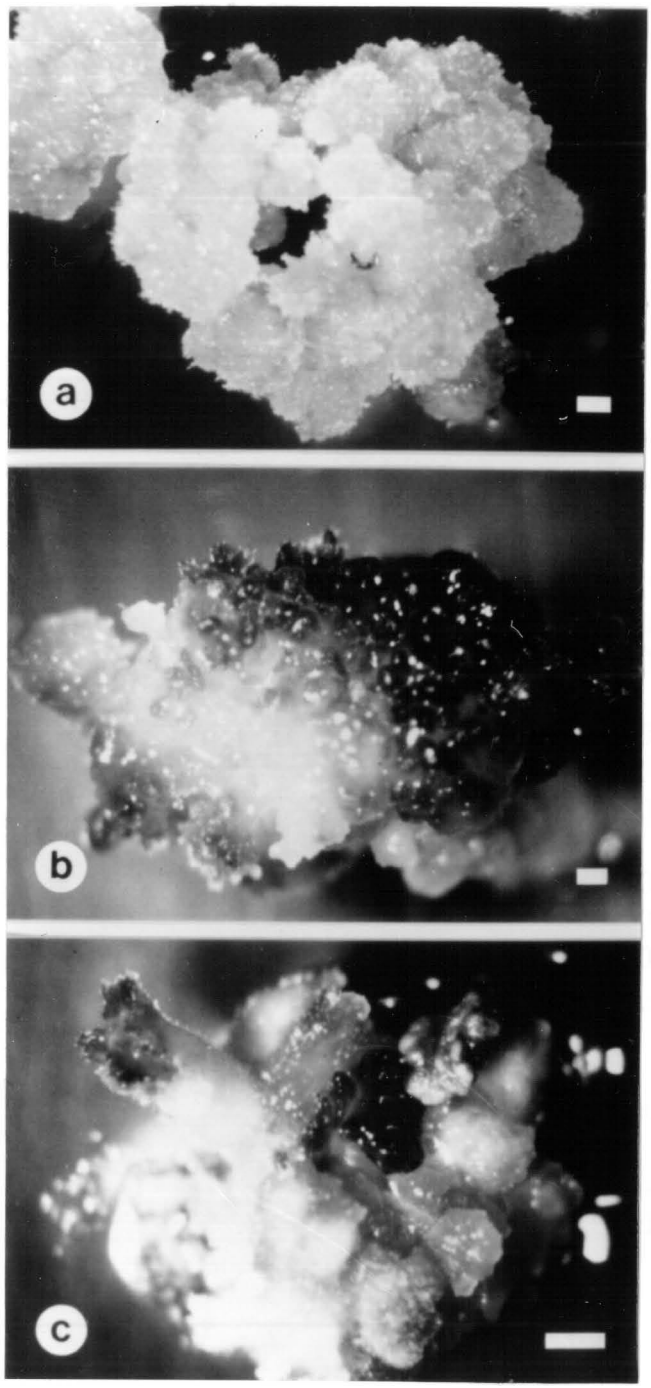

a
b
c

Table 3.1. Influence of growth regulators and dark pretreatment on callus development from petiole explants of *Cucumis metuliferus*.

Growth regulator combination[a]	Callus formation and appearance following incubation in: Dark		Light	
	Callus(%)[b]	Appearance[c]	Callus(%)[b]	Appearance[c]
IAA/BA	100	F, Y	100	P
IAA/Z	100	F, Y	100	P
IAA/K	100	W, R	100	P
NAA/BA	100	F ,Y	100	F, Y
NAA/Z	100	F, Y	100	F, Y
NAA/K	100	R, W	100	R, W
2,4-D/BA	100	F ,Y, D	100	C, Y, D
2,4-D/Z	100	F, Y	100	F, Y
2,4-D/K	100	F, R	100	F, Y, R

[a] Tested at a concentration of 1.0/1.0 μM.
[b] Within each treatment, a total of 32 explants were included.
[c] Appearance of callus rated as follows:
F=friable; C=compact; Y=yellow; W=yellowish white; R=rooting; D=differentiation; P=poor callus growth and browning.

Table 3.2. Influence of growth regulator combinations in callus induction medium and effect of subculture on differentiation of callus from petiole explants of *Cucumis metuliferus*

Growth regulator combination and concentration (μM)[a]	Callus formation (%)[b]	Percent differentiation after	
		No sub-culture	Onto 4.0 μM Z[c]
NAA/BA (2.0/1.0 μM)	100	0	3.9 ± 1.5
2,4-D/BA (2.0/1.0 μM)	100	0	11.8 ± 1.7

[a] In callus medium for 4 wk.
[b] Within each treatment, a total of 72-120 explants were included.
[c] Rated after 3 wk. Values represent the mean ± SE. The experiment was repeated twice.

Table 3.3. Influence of varying concentrations of two growth regulator combinations on callus differentiation and shoot formation from petiole explants of *Cucumis metuliferus*

Growth regulator combinations	Concentration (μM)	Callus with differentiation (%)[a]	Callus with shoots (%)[b]
2,4-D/BA	0.5/1.0	6.3 ± 2.4[c]	2.1 ± 1.4
	1.0/1.0	14.6 ± 4.6	8.3 ± 2.6
	2.0/1.0	20.8 ± 4.2	14.6± 3.4
	4.0/1.0	6.3 ± 2.9	0.0 ± 0.0
NAA/BA	0.5/1.0	2.5 ± 1.7	0.0 ± 0.0
	2.0/1.0	0.0 ± 0.0	0.0 ± 0.0

a Rated after 5 wk of incubation.

b Rated 2 wk after transfer of differentiating callus onto MS medium with 2.0 μM zeatin.

c Each value represents the mean ± SE of 10-12 replications, each with 8 explants, per treatment. The experiment was repeated twice.

3.4 Discussion

The results from this study have shown that regeneration of shoots from callused petiole explants of *C. metuliferus* can be achieved at a frequency of 14.6%. Previous attempts to regenerate plantlets from tissue cultures of this species were unsuccessful (Orczyk *et al.*, 1988; Punja *et al.*, 1990a). Callus formation from all explants tested in this study was prolific, confirming previous reports on the ease of callus induction in this species (Orczyk *et al.*, 1988; Punja *et al.*, 1990a). However, only callus from petiole explants subsequently differentiated to form shoot-like structures in this study. In a previous study of various cultivars of *C. sativus*, the highest regeneration frequencies were reported to occur also from petiole explants (Punja *et al.*, 1990b). A dark pretreatment of callus for 2 weeks did not subsequently enhance differentiation in *C. metuliferus*, although it was shown to significantly enhance regeneration in *C. sativus* (Punja *et al.*, 1990b).

In a previous study, which used leave explants and various combinations of auxins (IAA, NAA, 2,4-D, and 2,4,5-T) and cytokinins (2iP and BA) (Orczyk *et al.*, 1988), organogenesis occured with formation of adventitious roots (on NAA/2iP medium), but no bud or shoot formation was observed. In a preliminary study using different explant types (cotyledons, hypocotyl, leaves, petioles and roots) and various combinations of PGRs, prolific callus formation took place from most explants, but compact calli with visible signs of differenetiations were observed only on medium with 2,4-D and BA. However, if these calli were kept in the same

type of medium for more that 6 weeks, the differentiated portions of the calli started to undergo dedifferentiated. The important keys in the plantlet regeneration in this study, which differed from the two previous studies with C. *metuliferus*, were probably the development of differentiating and compact calli from the petiole explants to form adventitious buds or shoots (by organogenesis) on medium with 2,4-D and BA at low concentrations, and further shoot development on medium containing zeatin.

The procedure described in this study represents the first successful attempt at regeneration of plantlets from the wild African horned cucumber, C. *metuliferus*. The procedure may be applicable to regeneration from calli derived from protoplasts (McCarthy *et al.*, 1989; Dabauza *et al.*, 1991), and could have applicability in protoplast fusion studies (Punja and Raharjo, 1992), in which minicalli and calli may be obtained from plated protoplasts but regeneration of plantlets from the calli is still problematic.

CHAPTER IV

REGENERATION OF PLANTLETS FROM EMBRYOGENIC SUSPENSION CULTURES OF PICKLING CUCUMBER (*CUCUMIS SATIVUS* L.) CV. ENDEAVOR

4.1 Introduction

The ability to achieve high potentials of plantlet regeneration from cultured cells or tissues is a prerequisite for the application of tissue culture procedures for crop improvement. Methods for high frequency plantlet regeneration from calli of fresh market and pickling cucumber cultivars via embryogenesis or organogenesis have recently been described (e.g.. Chee, 1990b; Punja *et al.*, 1990b). The optimal conditions for achieving high regeneration frequencies were developed by manipulation of plant growth regulator combinations and concentrations, and by selecting the most suitable explant sources. These conditions were found to vary for different cultivars of cucumber (Punja *et al.*, 1990b) and for other *Cucumis* species (Punja *et al.*, 1990a; Chapter III).

Plantlet regeneration has been achieved from cell suspension cultures of several fresh market cultivars of cucumber (Malepszy and Solarek, 1986; Chee and Tricoli, 1988; Bergervoet *et al.*, 1989). Various methods for the initiation of cell suspension cultures, production of embryos, and regeneration of plantlets of these fresh market cultivars have

been described. Chee and Tricoli (1988) achieved a high frequency of regeneration of normal plantlets from cell suspension cultures via embryogenesis by washing the cells with MS medium containing activated charcoal prior to embryo development in liquid MS basal medium, and later plating onto conversion medium. In another study (Ziv and Gadasi, 1986), plantlet regeneration via embryogenesis was attained by using a liquid/solid double layer system containing charcoal. High regeneration of normal plantlets was achieved also through bud formation (organogenesis) after suspension aggregates in medium with NAA and BA were plated onto solid medium containing a low concentration of IAA and kinetin (Bergervoet *et al.*, 1989).

The objective of this study was to develop procedures for establishment and maintenance of suspension cultures of the pickling cucumber cultivar Endeavor, and for regeneration of normal appearing plantlets at a high frequency from the suspension cultures. The effects of various concentrations of auxin and cytokinin in the plating medium, the use of $AgNO_3$, and various methods of plating the cell aggregates, were evaluated.

4.2 Materials and Methods

4.2.1 Plant material

The pickling cucumber cultivar Endeavor (seed provided by Campbell Seeds, Davis, CA), a gynoecious hybrid (WI 2870G x Clinton), was

used in this study. Procedures for preparation of sterile seedlings as explant sources were the same as those previously described (Chapter II, subsection 2.2.1).

4.2.2 Initiation of callus

Petiole segments, 3-5 mm in length, from 10- to 16-d-old *in vitro*-grown seedlings were used as the explant source. The medium used for callus initiation was full-strength MS medium, with a full complement of major and minor salts, vitamins, 30 $g \cdot l^{-1}$ sucrose and with 100 $mg \cdot l^{-1}$ ampicillin. The medium was supplemented with 2,4-D/BA (5.0/5.0 μM) and solidified by addition of 10 $mg \cdot l^{-1}$ Sigma tissue culture agar. The pH was adjusted to 5.8 prior to autoclaving at 121°C and 1.05 $kg \cdot cm^2$ for 20 min. Approximately 25 ml of medium was dispensed into disposable Petri dishes (20x100 mm). Eight explants were placed in each Petri dish, and the dishes were sealed with Parafilm®. All dishes were incubated in continuous darkness for 3 weeks and transferred to the light (provided by cool-white fluorescent lamps, intensity of 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at culture level), a 16-$h \cdot d^{-1}$ photoperiod, at ambient temperatures of 25-28°C.

4.2.3 Initiation of suspension cultures

Eight weeks after the explants were plated, creamy-yellow embryogenic calli which developed from the explants (Fig. 4.1 a) were dissected and used as the donor of cells or cell aggregates for suspension culture. Liquid MS medium containing full strength major and minor salts, vitamins, and 30 $g \cdot l^{-1}$ sucrose was used. The following combinations

of growth regulators were evaluated: NAA/BA (1.0/1.0 μM), NAA/Z (1.0/1.0 μM), NAA/adenine-SO_4 (1.0/200 μM), 2,4-D/BA (1.0/1.0 μM), and 2,4-D (1.0 μM). Embryogenic callus (1-2 g fresh weight) from one explant was added to each 250 ml Erlenmeyer flask containing 75 ml of medium. There were three flasks for each combination of growth regulator tested. The flasks were kept in dim light at ambient temperatures of 24-28°C and shaken continuously on a gyratory shaker at 120 rpm. After 4 weeks, the condition of the suspension cultures was noted and healthy-appearing cultures were subsequently subcultured into fresh medium containing the same combinations of growth regulators. This first subculture was carried out following sieving of the suspension culture through Sigma cell sieve (with 0.38 mm openings), followed by two rinses of the cells/aggregates using liquid MS medium without growth regulators, and then transferring the tissue into the fresh medium.

4.2.4 Maintenance of suspension cultures

Two weeks after the first subculture, suspension cultures initiated in medium with 2,4-D/BA (1.0/1.0 μM) showed the best appearance, i.e. produced a large number of yellow cells/aggregates, did not form roots or embryo elongation and did not show evidence of browning. Only these suspension cultures were maintained further through subcultures every 2 weeks as follows. The flasks were removed from the shaker and the cells/aggregates were allowed to settle to the bottom of the flask. About 10 ml of the suspension (with dense cells/aggregates) was pipeted (using a volumetric pipette of 10 ml capacity with a large-holed tip) into 75 ml of

fresh medium with 2,4-D/BA (1.0/1.0 μM) and the flask was shaken manually. After the cells/aggregates had settled, 2.0 ml was pipeted from the bottom and transferred into a flask containing 75 ml of fresh maintenance medium with 2,4-D/BA (1.0/1.0 μM). The suspension cultures were maintained on a gyratory shaker at 120 rpm, at the same conditions as those for their initiation.

4.2.5 Regeneration

Experiment 1. The first attempt to induce regeneration was carried out using five-month-old cultures by plating one ml of the suspension, 2 weeks after last subculture, onto MS medium without any growth regulators (MS0), or MS0 with 0.5% activated charcoal (Sigma Chemical Co., St. Louis, MO), or five 100 μL drops were plated on MS with 1.0 μM NAA. However, these procedures did not induce formation of normal plantlets (authors, unpublished). A second procedure similar to that described by Bergervoet *et al.* (1988) was tested using 11-month-old cultures. All the cells/aggregates from each flask, 10 d after the last subculture, were pipeted (using a large-holed pipette) into another flask containing 100 ml liquid MS medium without growth regulators and shaken manually. After all of the cells/aggregates had settled, approximately 10 ml of the medium with dense aggregates was pipeted out. To plate the aggregates, five 100 μL drops of the suspension were plated onto filter paper (Whatman) overlaid onto solid regeneration medium containing either IAA/kinetin (0.1/1.0 μM) or NAA/BA (2.0/1.0 μM). For each treatment, three or four replicate Petri

dishes were included, and the experiment was conducted twice. The data presented are the average across all replications.

Experiment 2. This experiment was intended to find the optimal combinations of auxins (2,4-D or NAA) and cytokinin (BA) and to test the effects of $AgNO_3$ for embryo or shoot regeneration after plating embryogenic suspension culture onto solid medium. Three-month-old embryogenic suspension cultures were used. Cells/aggregates from each flask, 1 week after the last subculture, were pipeted and transferred into another flask containing 50 ml of liquid MS medium without growth regulator and shaken manually. After all of the cells/aggregates had settled, approximately 5.0 ml of the medium with dense aggregates was pipeted out. To plate the aggregates, nine 60 µL drops of the suspension were plated onto filter paper overlaid on solid MS medium containing 2,4-D/BA (1.0/1.0 µM), 2,4-D/BA (5.0/5.0 µM), NAA/BA (1.0/1.0 µM) or NAA/BA (5.0/5.0 µM). The medium used was either with or without 30 µM $AgNO_3$ which was filter-sterilized and added after autoclaving. For each treatment, three or four replicate Petri dishes were included, and the experiment was conducted twice. The data presented are the mean of all replications.

4.3 Results

Petiole segments initiated callus after about 1 week on medium containing 2,4-D/BA (5.0/5.0 µM) and the callus developed embryogenic (yellow and friable) sectors and embryos 5-7 weeks later (Fig. 4.1 a). This embryogenic callus was dissected and transferred into liquid medium to

initiate the suspension culture. After 2-3 weeks of shaking on a gyratory shaker, callus in the medium with 2,4-D/BA (1.0/1.0 μM) started to break apart, forming a suspension of cells/aggregates (Fig. 4.1 b).

Experiment 1. In the medium containing either NAA/BA (1.0/1.0 μM), NAA/Z (1.0/1.0 μM), NAA/adenine-SO_4 (1.0/200 μM), or 2,4-D (1.0 μM), the callus either did not break apart or formed elongated and rooted embryos, which resulted in browning of the tissue or the medium after 4 weeks. The medium containing 2,4D/BA (1.0/1.0 μM) appeared to be most appropriate for long-term maintenance of the suspension culture. Using this medium, healthy and repetitively embryogenic suspension cultures have been maintained in the laboratory for more than 15 months. A subculture every 2 weeks was optimal for long-term maintenance of the suspension culture. After more than 3 weeks, the aggregates increased in size and developed elongated and rooted embryos, which resulted in browning of the medium and a rapid decline of the culture quality. Aggregates from healthy suspension cultures were able to form normal shoots when plated onto filter paper overlaid on medium containing NAA/BA (2.0/1.0 μM). The frequencies of plantlet formation are given in Table 4.1. About 21 plantlets were obtained from each Petri dish plated with 500 μL of the initial suspension.

Experiment 2. Suspension-derived aggregates continued to develop friable yellow embryogenic calli (Fig. 4.1 c), and showed green sectors with developing somatic embryos (Fig. 4.1 d) or shoot primordia (Fig. 4.2 e) which appeared 1 - 2 weeks after plating on solid medium. The use of

regeneration medium with different concentrations of 2,4-D/BA or NAA/BA, either with or without $AgNO_3$, showed that the highest frequency of embryo and shoot development was achieved using NAA/BA (1.0/1.0 μM) without $AgNO_3$ (Table 4.2). The use of $AgNO_3$ at 30 μM in the regeneration medium did not consistently increase the capacity of embryo or shoot development, or even reduced it when a low level of NAA/BA or 2,4-D/BA (1.0/1.0 μM) was used.

Shoots which developed on regeneration medium from both experiments 1 and 2 (Fig. 4.1 f) were excised after 3 weeks and transferred onto MS medium without growth regulators, where they elongated and grew larger (Fig. 4.1 g), and finally developed roots after two to three subcultures onto the same medium. Calli which occasionally grew on the bottom parts of the plantlets were removed to enhance normal growth. Although the first leaves appeared distorted, subsequent leaves usually looked normal. Upon transfer into soil and maintaining in a growth chamber at 26°C and a 16-h•d^{-1} photoperiod, the rooted plantlets yielded plants which appeared normal (Fig. 4.1 h) and which flowered and set fruits.

Figure 4.1. Initiation of embryogenic suspension cultures of pickling cucumber and regeneration of normal plants. **a)** Embryogenic calli growing on MS medium with 2,4-D/BA (5.0/5.0 μM) 5 weeks after plating of petiole explants (bar = 2 mm); **b)** Embryogenic cell aggregates developing in suspension culture with 2,4-D/BA (1.0/1.0 μM), 5 weeks after initiation; **c)** Differentiation of droplets of embryogenic aggregates plated on medium with NAA/BA (1.0/1.0 μM) (bar = 2 mm); **d)** Development of embryogenic calli and embryos 3 weeks after subculturing callus from droplets onto MS medium with 2,4-D/BA (1.0/1.0 μM) (bar = 2 mm); **e)** Shoot development on callus (bar = 1 mm); **f)** Further development of shoots on MS0 medium; **g)** Plantlets with normal leaves and roots on MS0 medium; **h)** Two-week-old (front row) and 4-week-old (rear) plants after transfer of plantlets into pots containing soil.

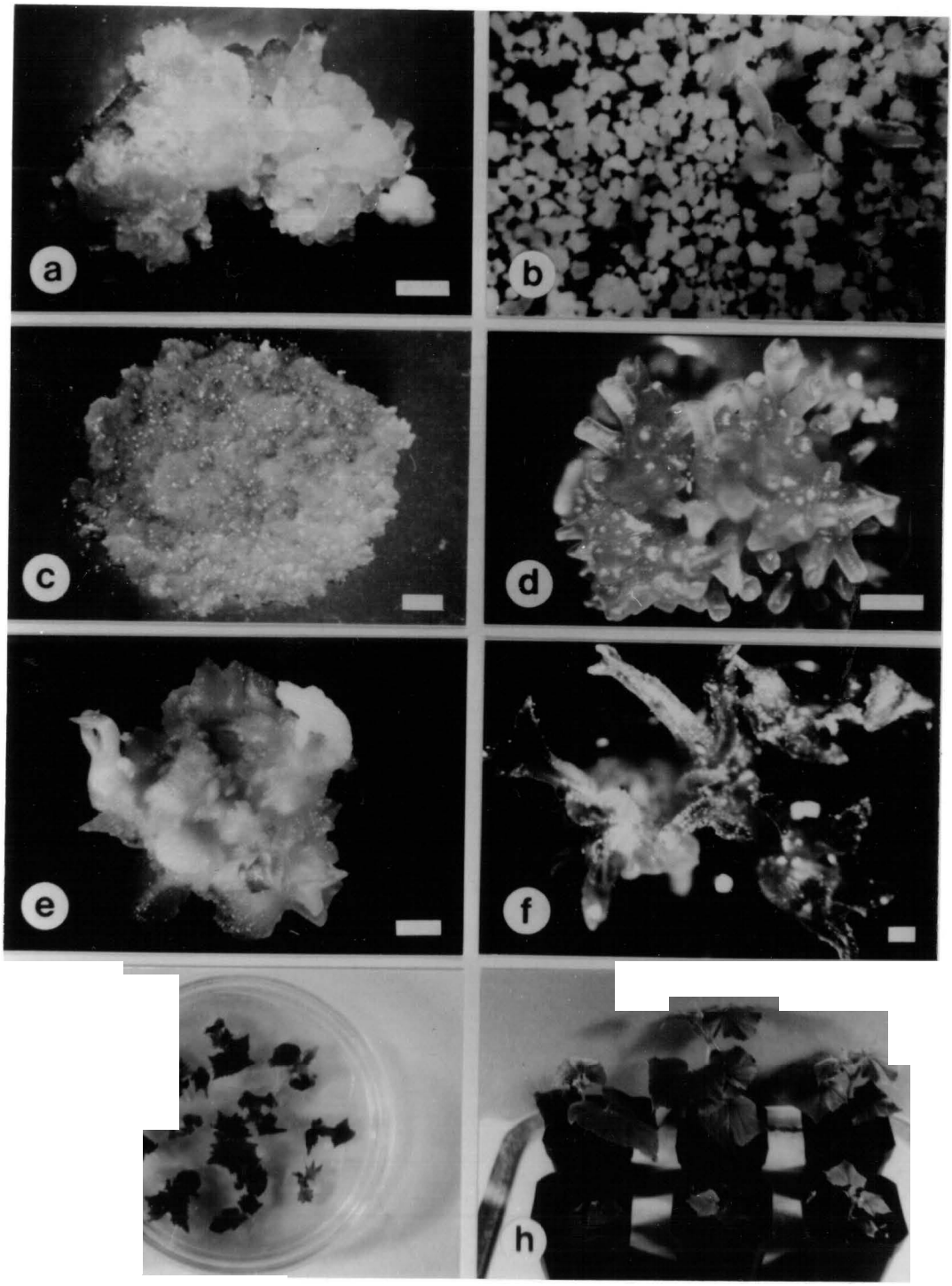

a
b
c
d
e
f
h

Table 4.1. Frequency of regeneration and appearance of plantlets of cucumber cv. Endeavor 3 weeks after plating aggregates from suspension cultures

Plating medium	Number of plantlets per dish[a]		Appearance
	Total	Normal	
MS0[b]	0	0	aggregates forming calli, embryo dedifferentiation, rooting, browning
MS + charcoal (0.5%)[b]	0	0	aggregates forming calli, embryo dedifferentiation, rooting, browning
MS + NAA (1μM)[c]	18	0	aggregates forming calli and shoots, shoot dedifferentiation
MS + NAA/BA (2.0/1.0 μM)[d]	39	21	aggregates forming calli and normal shoots from embryos, less shoot dedifferentiation, rooting
MS + IAA/kinetin (0.1/1.0 μM)[d]	35	0	aggregates forming calli and shoots, shoot dedifferentiation, rooting

[a] Mean of three to eight dishes; the experiment was conducted twice

[b] One ml of suspension culture was plated onto each dish of medium, 14 d after the last subculture

[c] Five 100 μL drops of suspension culture were plated onto each dish of medium, 10 d after the last subculture

[d] Five 100μL drops of dense cell/aggregate suspension were plated onto filter paper overlaid onto medium, 10 d after the last subculture

Table 4.2. The effects of different concentrations of 2,4-D/BA and presence of $AgNO_3$ in the plating medium on plantlet regeneration from suspension cultures of cucumber cv. Endeavor

Plating Medium	Development of Embryogenic Calli (%)[a]	Number of Embryos/ Shoots per Plating Dish[b]	Frequency of Normal Plantlets (%)
A. Without $AgNO_3$			
NAA/BA (1.0/1.0 μM)	100	30.8 (±3.5)	42
NAA/BA (5.0/5.0 μM)	100	23.8 (±2.1)	0
2,4-D/BA (1.0/1.0 μM)	100	18.8 (±1.4)	41
2,4-D/BA (5.0/5.0 μM)	100	9.8 (±1.7)	13
B. With $AgNO_3$ (30 μM)			
NAA/BA (1.0/1.0 μM)	84	14.0 (±2.0)	46
NAA/BA (5.0/5.0 μM)	96	26.2 (±2.4)	29
2,4-D/BA (1.0/1.0 μM)	87	11.0 (±0.9)	38
2,4-D/BA (5.0/5.0 μM)	100	14.4 (±1.7)	21

a Rated 2 weeks after plating; based on the number of drops of aggregates which formed embryogenic calli over total drops in each treatment.

b Rated 3 weeks after plating; each value represents the mean (±SE) of six replications, each with nine drops of aggregates per dish; the experiment was conducted twice.

4.4 Discussion

The addition of activated charcoal, which was useful for initiating regeneration of plantlets from callus as well as from suspension cultures in other studies (Ziv and Gadasi, 1986; Chee and Tricoli, 1988), was not effective in our study. This may have been due to the fact that our suspension cultures had been maintained over a longer period of time, or possibly to genotype differences. Cells/aggregates plated onto MS medium with IAA/kinetin (0.1/1.0 μM) developed adventitious shoots through organogenesis, but the shoots dedifferentiated rapidly prior to the time where they could be transferred to MS0 for plantlet development. The use of NAA/BA at 2.0/1.0 μM in the plating medium provided the highest recovery of plantlets in our study. The addition of $AgNO_3$ at 30 μM increased regeneration capacity slightly only when a higher concentration of NAA/BA or 2,4-D/BA, i.e. 5.0/5.0 μM, was used. At lower concentrations of NAA/BA (1.0/1.0 μM), $AgNO_3$ reduced the capacity of regeneration. While $AgNO_3$, which inhibits ethylene action, has been shown to enhance regeneration in tissue culture of several other crops (De Block, 1988; Palmer, 1992; Roustan *et al.*, 1992), it did not enhance recovery of plantlets of pickling cucumber in this study.

Results from this work using a pickling cucumber cultivar add to the information previously published on repetitive formation of embryogenic aggregates in liquid culture of fresh market cucumber cultivars (Chee and Tricoli, 1988; Bergervoet *et al.*, 1989), and also describe new procedures for this type of cucumber. In this study, embryogenic calli were used to initiate

the suspension cultures and a relatively low concentration of growth regulators (i.e. 2,4-D/BA at 1.0/1.0 μM) was used. Regenerable aggregates could be maintained in suspension culture for as long as 15 months by subculturing every 2 weeks. Furthermore, since embryogenic aggregates can be produced rapidly and can be plated repeatedly once established, plantlets may be produced at a high capacity through clonal propagation within a short period of time. The procedure developed will be applicable to propagation of individual calli into large numbers of plantlets, and can be used to facilitate the selection of transformants in our genetic transformation work, in which the direct regeneration of plantlets from putatively transformed individual calli is difficult (Sarmento *et al.*, 1992). Liquid embryogenic cultures have been utilized also for gene delivery or transformant selection in transformation studies of *Glycine max* (McMullen and Finer, 1991), *Zea mays* (Gordon-Kamm *et al.*, 1990), *Gossypium hirsutum* (Finer and McMullen, 1990), and *Mangifera indica* (Mathews *et al.*, 1992).

CHAPTER V

INTRODUCTION OF CHITINASE-ENCODING GENES INTO PICKLING CUCUMBER BY *AGROBACTERIUM TUMEFACIENS*-MEDIATED TRANSFORMATION

5.1 Introduction

An important application of cucumber (*Cucumis sativus* L.) tissue and cell culture is for plant improvement through genetic engineering (Trulson and Shahin, 1986; Chee and Slightom, 1991; Sarmento *et al.*, 1992). Several procedures to regenerate plantlets from cucumber tissue and cell cultures have been developed for fresh market and pickling cultivars (Chee and Tricoli, 1988; Orczyk *et al.*, 1988; Cade *et al.*, 1990; Chee, 1990b; Gambley and Dodd, 1990; Punja *et al.*, 1990b; Chapter IV). Cucumber is naturally susceptible to *Agrobacterium tumefaciens* (Anderson and Moore, 1979). However, the application of *Agrobacterium*-based transformation systems for foreign gene transfer into cucumber has only been demonstrated in a few studies. The gene encoding kanamycin resistance (Chee, 1990a; Sarmento *et al.*, 1992) and a coat protein gene conferring virus resistance (Chee and Slightom, 1991) have been expressed in transgenic cucumber plants. However, except for virus resistance, other potentially useful agronomic traits have not been engineered into cucumber.

Chitinases are PR proteins which have been implicated in plant defense against fungal infection (Boller *et al.*, 1983; Punja and Zhang, 1993). The substrate is chitin, which does not occur in plants but which is an important component of fungal cell walls (Linthorst *et al.*, 1990). The acidic and basic chitinases differ in isoelectric points, in the subcellular targeting of the enzyme, and in antifungal activity (Punja and Zhang, 1993). In cucumber, the indigenous chitinase is acidic and is induced by various factors, such as pathogen infection and other biotic and abiotic stresses (Metraux, 1986; Zhang and Punja, 1993). The introduction of genes encoding chitinases into cucumber would be useful to elucidate the role of different chitinases in plant disease resistance to filamentous fungal pathogens.

The objective of this study was to introduce a heterologous acidic chitinase gene cloned from petunia (Linthorst *et al.*, 1990), and a basic chitinase gene cloned from tobacco (Linthorst *et al.*, 1990) and bean (Broglie *et al.*, 1991), into pickling cucumber using *Agrobacterium*. The integration of the chitinase genes and expression of the proteins in transgenic plants are described.

5.2 Materials and Methods

5.2.1 Plant material

Cucumber cultivar and the procedures for preparation of sterile plant material used in this genetic transformation study are the same to

those in Chapter IV subsection 4.2.1. Explants used were from petioles (4-5 mm^2 segments) taken from the first and second true leaves of 10- to 21-d-old seedlings.

5.2.2 Bacterial strains

Three *A. tumefaciens* strains with different Ti helper plasmids were used: a) EHA105 (a supervirulent leucinopine type), b) MOG101 (an octopine type), and c) MOG301 (a nopaline type). The strains were provided by Dr. L. Melchers (Mogen Int. nv, The Netherlands) and harboured one of two binary vectors (pMOG196 and pMOG198) containing the petunia acidic chitinase gene and the tobacco basic chitinase gene, respectively (Linthorst *et al.*, 1990). A third vector, pGA492-CHN (Hernandez, 1994), containing a bean basic chitinase gene (*CH5B*), was designed by ligation of the 2.5 kb *EcoRI-ClaI* fragment of pk35CHN-641, provided by Dr. R. Broglie (E.I. DuPont Agricultural Products, Wilmington, DE) (Broglie *et al.*, 1991) into a *EcoRI-ClaI* linearized plasmid pGA492 (An, 1987). The vector was transferred into each of the three strains by triparental mating (Turk *et al.*, 1991). All vectors contained the *NPT II* gene as a selectable marker and the CaMV *35S* promoter for constitutive expression.

5.2.3 Preparation of bacterial suspension

The *Agrobacterium* strains were grown on LB and Minimal media (Turk *et al.*, 1991) with 100 $mg \cdot l^{-1}$ kanamycin, pH 5.4. The procedure for preparation of bacterial suspension was as follows. A single colony was

picked and inoculated into LB medium containing 100 $mg \cdot l^{-1}$ kanamycin (10-20 ml of liquid medium in a 250 ml Erlenmeyer flask). The culture was incubated for 24 h on a shaker at 120 rpm which was kept in an incubator set at 29°C. Subsequently, the bacterial culture was diluted (1 : 50) into 20-25 ml of minimal medium with 100 $mg \cdot l^{-1}$ kanamycin and incubated for 24 h under the same conditions as previously. Before being used for infection of explants, the bacterial suspension was rinsed twice using MS medium (pH 5.4) by centrifuging at approximately 900 x *g*. The bacterial pellet was suspended in MS medium and the suspension was adjusted to a final concentration of about 10^8 $cells \cdot ml^{-1}$ as determined by counts in a haemocytometer. One hour before infection of explants, acetosyringone was added to a final concentration of 100 µM.

5.2.4 Infection of explants and cocultivation

Infection was carried out by dipping 150 to 240 explants per treatment, which were sectioned approximately 1-2 h prior to infection, into the bacterial suspension for 5 min (each of the three strains were evaluated in separate experiments), followed by blotting the explants on sterile paper towels. The explants were subsequently transferred onto cocultivation medium, consisting of MS salts and other ingredients, with 2,4D/BA (5.0/5.0 µM), at pH 5.4, without antibiotics, in disposable Petri dishes (20x100 mm). Cocultivation was carried out by incubating the dishes at 27°C in the dark for 2-4 d.

5.2.5 Selection of transformed calli

After cocultivation, explants were washed three times by rinsing with MS medium, with the last rinse containing 500 $mg \cdot l^{-1}$ ampicillin, and blotting them on sterile paper towels. The cocultivated explants were placed on selective medium, consisting of MS salts and other ingredients, with 2,4-D/BA (5.0/5.0 μM), kanamycin (50 $mg \cdot l^{-1}$) and carbenicillin (500 $mg \cdot l^{-1}$). The two antibiotics were added to inhibit untransformed cells and to kill *Agrobacterium*, respectively. The kanamycin concentration of 50 $mg \cdot l^{-1}$ was selected based on our previous study in which no somatic embryogenesis occurred at kanamycin concentrations of 40 $mg \cdot l^{-1}$ or higher (Chapter II). Dishes were incubated in the dark at 24-26°C for 4 weeks. Controls were included in these transformation experiments, and consisted of a positive control (no cocultivation, no antibiotics in callus initiation medium) and a negative control (no cocultivation, using selective medium with kanamycin and carbenicillin). Explants and calli were subcultured to fresh medium of the same composition 4 - 5 weeks after infection. Response of the explants to the treatments was recorded 8 weeks after infection and data collected included callus formation, explants+callus diameter, and presence of bacterial ooze. Transformation efficiencies were determined based on the number of explants which produced kanamycin-resistant embryogenic calli per total explants after cocultivation in an experimental treatment.

Embryogenic calli obtained after one or two subcultures were used to initiate a suspension culture (summarized in Appendix IV). The purpose

of this suspension culture was to further screen for kanamycin resistant cell aggregates and to multiply them. Procedures for initiation and maintenance of these suspension cultures have been described (Chapter IV). Liquid MS medium containing full strength major and minor salts, vitamins, 30 $g \cdot l^{-1}$ sucrose, and supplemented with 2,4-D/BA (1.0/1.0 µM) and kanamycin (50 $mg \cdot l^{-1}$), was used. To initiate the suspension culture, creamy-yellow embryogenic calli which developed from the explants were dissected and added to a 250 ml Erlenmeyer flask containing 75 ml of medium. The flasks were kept in dim light (≈30 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at ambient temperatures of 24-28°C and shaken continuously on a gyratory shaker at 120 rpm. The suspension cultures were maintained by subcultures every 2 weeks.

5.2.6 Regeneration of plantlets

Before plating for plantlet regeneration, cell aggregates and clumps (1 week after the last subculture) were rinsed with MS0 medium. The aggregates of various sizes (ranging from 1 to 12 mm in diameter) were plated onto solid MS medium 2,4-D/BA (1.0/1.0 µM) or NAA/BA (1.0/1.0 µM), supplemented with 50 $mg \cdot l^{-1}$ kanamycin. Shoots which developed on the regeneration medium were excised after 3 weeks and transferred to MS medium containing kanamycin but without growth regulators, where they grew and developed roots after 2-3 subsequent transfers onto the same medium. These plantlets were transferred into sterile potting mix (Sunshine Mix 1, Fisons Hort. Inc., Vancouver, BC) and were maintained in a Conviron growth chamber (with fluorescent lighting,

≈40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at plant level) set at 26°C and a photoperiod of 16 $h \cdot d^{-1}$, where they developed into plants.

5.2.7 Analyses of transformed plants

Genomic DNA isolation and PCR. Total nucleic acids were extracted from putative transgenic plants and from untransformed (negative) controls using a procedure modified from Mettler (1987). Leaf tissue (100-300 mg) was cut and frozen in liquid nitrogen and ground to a fine powder in a small mortar. The powder was homogenized in buffer (1% SDS, 0.25 mM glucose, 50 mM NaCl, 20 mM EDTA, and 50 mM Tris, pH 8.0) and transferred to 1.5-ml centrifuge tubes. Following a 30-min incubation at room temperature, the DNA was extracted with an equal volume of TE saturated phenol (pH 8.0). The aqueous phase was repeatedly extracted with chloroform : isoamylalcohol (24 : 1) (Sevag) until the interface was clear. The genomic DNA was precipitated at -20°C with 0.3 M sodium acetate (pH 5.3) and two volumes of 95% ethanol. The nucleic acids were recovered by centrifugation for 20 min at 16,000 x *g*, at 4°C. The pellet was resuspended and incubated for 2 h at 37°C with 50 $\mu g \cdot ml^{-1}$ of RNAse. The DNA was then re-extracted with phenol : chloroform : isoamylalcohol (25 : 24 : 1), ethanol precipitated and resuspended in TE buffer. DNA concentrations were estimated by comparing with standards of herring sperm DNA.

Two specific primer sequences for the *NPT II* coding region, obtained from Dr. M.M. Moloney (University of Calgary, AB), were used for PCR

amplification of this gene in genomic DNA isolated from putatively transformed plants. Oligomer A, a 17mer with 5'-3' sequence GATGGATT-GCACGCAGG, is located 15 bp upstream from the start codon and oligomer B, a 17mer with 5'-3' (bottom strand) sequence GAAGGCGATAGAAGGCG shares identity with the 3' region of the *NPT II* gene, 17 bp 5' of the stop codon. Each PCR reaction (25 µl overlaid by 50 µl of mineral oil) consisted of 1X *Taq* buffer ($MgCl_2$-free), 1 mM $MgCl_2$, 200 µM of dNTPs, 0.5 µM of each oligo-nucleotide primer, and 100-200 ng of template DNA. Each PCR reaction was incubated at 92°C for 5-10 min and 1.25 units of *Taq* polymerase (Promega) was added and quickly placed on ice. The temperature cycling for the PCR was as follows: 29 cycles of 94°C for 1 min, 54°C for 2 min and 72°C for 3 min. The 30th cycle was the same with the exception that DNA extension at 72°C was carried out for 10 min. The PCR products were then analysed by electrophoresis on 2.0% agarose gels. The oligomers were first tested by amplifying the characteristic 800 bp region of the *NPT II* gene by using 200 ng of pMOG196 as positive control template.

Southern analysis. Approximately 12 µg of genomic DNA was digested with *HindIII* and the fragments were separated by electrophoresis on 0.7% agarose gels. DNA fragments were transferred from the gels to positively-charged nylon membranes (Boehringer Mannheim, Laval, PQ) by capillary blotting with 20 X SSC and fixed by UV crosslinking. The probe was made by amplification of the 800 bp fragment from the vector which contained the *NPT II* coding region using the PCR conditions specified above, and was labeled with Digoxigenin-UTP. Hybridization was conducted using about 15 ng of the probe per ml of hybridization solution

containing 2% blocking buffer (Boehringer Mannheim, Laval, PQ) and 50% formamide and incubated at 40-42°C for at least 15 h. Hybridization filters were washed and detected using chemiluminescence according to manufacturer's instructions. The blots were finally exposed to Kodak OMAT-K X-ray film for 24 h.

Western and immuno-dot blot analyses of chitinase and assay for chitinase activity. Young leaves from transgenic cucumber plants were frozen in liquid nitrogen immediately after collection, and finely ground into a powder using a mortar and pestle. All extractions were performed at 4°C. The powder was extracted with 0.1 M sodium citrate buffer (pH 5.0), and the crude homogenate was filtered through four layers of Miracloth and centrifuged at 20,000 x *g* for 30 min. The supernatant was transferred onto an ultrafiltration unit (Amicon YM10 filter) for adjustment of protein concentrations. Protein samples were loaded and separated by SDS-PAGE. The separated samples were transferred to 0.45 µm nitrocellulose membranes at 5 $mA.cm^{-2}$ for 30 min in Bjerrum and Schafer-Nielsen transfer buffer with a Bio-Rad Trans-Blot semi-dry electrophoretic transfer cell as described in the instruction manual. Western blots were performed following the procedure described in the Bio-Rad Immuno-Blot GAR-AP assay kit instruction manual. The antiserum raised against petunia chitinase, provided by Dr. L. Melchers (Mogen International nv, The Netherlands) was used at 1 : 5,000 dilution for assay of transgenic plants with a petunia chitinase transgene. The antisera raised against tobacco chitinase, provided by Dr. B. Fritig (Institute de Biologie Moleculaire des Plantes, France) and bean chitinase, provided by Dr. R. Broglie (E.I. DuPont

Agricultural Products, USA) were used at 1 : 2,000 dilution for assays of transgenic plants expressing tobacco and bean chitinase transgenes, respectively. A quantitative assay of endochitinase activity in transgenic and control plants was conducted using a modified colorimetric method as described by Legrand et al. (1987). In this assay, samples from four transgenic plants transformed using pMOG196 with a tobacco acidic chitinase gene, i.e. plants #2, #14, #19 and #21, and from one control plant, were used.

5.3 Results

5.3.1 Development of kanamycin-resistant embryogenic calli

Cocultivated petiole segments swelled and formed callus after 3 - 4 weeks on selective callus initiation medium containing 2,4-D/BA (5.0/5.0 μM), kanamycin (50 $mg \cdot l^{-1}$) and carbenicillin (500 $mg \cdot l^{-1}$) (Fig 5.1 a). Calli which survived this selection step were subcultured to fresh medium of the same composition where they developed embryogenic (yellow and friable) sectors (Fig 5.1 b). By comparison, most non-cocultivated explants were bleached and did not develop further. The capacity of embryogenic calli which developed further and grew on kanamycin-containing medium, recorded 8 weeks after cocultivation, ranged from 0 to 12%, depending on *Agrobacterium* strain and vector used (Fig. 5.2). The highest capacity (12%) was achieved when strain EHA105 (supervirulent leucinopine) with pMOG196 was used. The length of cocultivation, either 2 or 4 d, did not

appear to consistently affect the capacity of development of kanamycin-resistant embryogenic calli. No direct shoot formation via embryogenesis or organogenesis was obtained from these calli over a 3-month period.

5.3.2 Suspension culture and development of plantlets

The embryogenic sectors from kanamycin-resistant calli were dissected and transferred into liquid medium containing 2,4-D/BA (1.0/1.0 μM) and kanamycin (50 $mg \cdot l^{-1}$) to initiate the suspension culture. Kanamycin-resistant embryogenic aggregates could be maintained by a subculture every 7-10 d. Embryogenic calli obtained from non-cocultivated explants did not develop when transferred into the liquid medium containing kanamycin. Shoots were obtained by plating kanamycin-resistant embryogenic aggregates onto solid medium containing 2,4-D/BA (1.0/1.0 μM) and kanamycin (50 $mg \cdot l^{-1}$) (Figs 5.1 c and d). When the shoots were excised after 4 - 5 weeks and transferred onto MS medium without growth regulators and with kanamycin (50 $mg \cdot l^{-1}$), they elongated and developed roots after 2 - 3 subcultures onto the same medium (Figs 5.1 e and d). Upon transfer into soil and maintaining in a growth chamber at 26°C and a photoperiod of 16 $h \cdot d^{-1}$, the rooted plantlets developed into plants. All transgenic plants were obtained using strain EHA105 (supervirulent). A total of 32 plants were obtained following transformation with pMOG196, 9 plants with pMOG198, and 44 plants with pGA492-CHN.

5.3.4 Confirmation of transformation

PCR and Southern analysis. DNA was isolated from plants that were regenerated from kanamycin-resistant calli as well as from control (untransformed) plants. Evidence of transformation was confirmed by PCR amplification of the *NPT II* gene using two specific primer sequences for the *NPT II* coding region. Three randomly selected plants, each transformed with one of pMOG196, pMOG198 and pGA492-CHN, and each originating from one transformation event, produced bands of the expected size of 800 bp for the *NPT II* fragment at the same position as the binary vector positive control (pMOG196) (Fig 5.3).

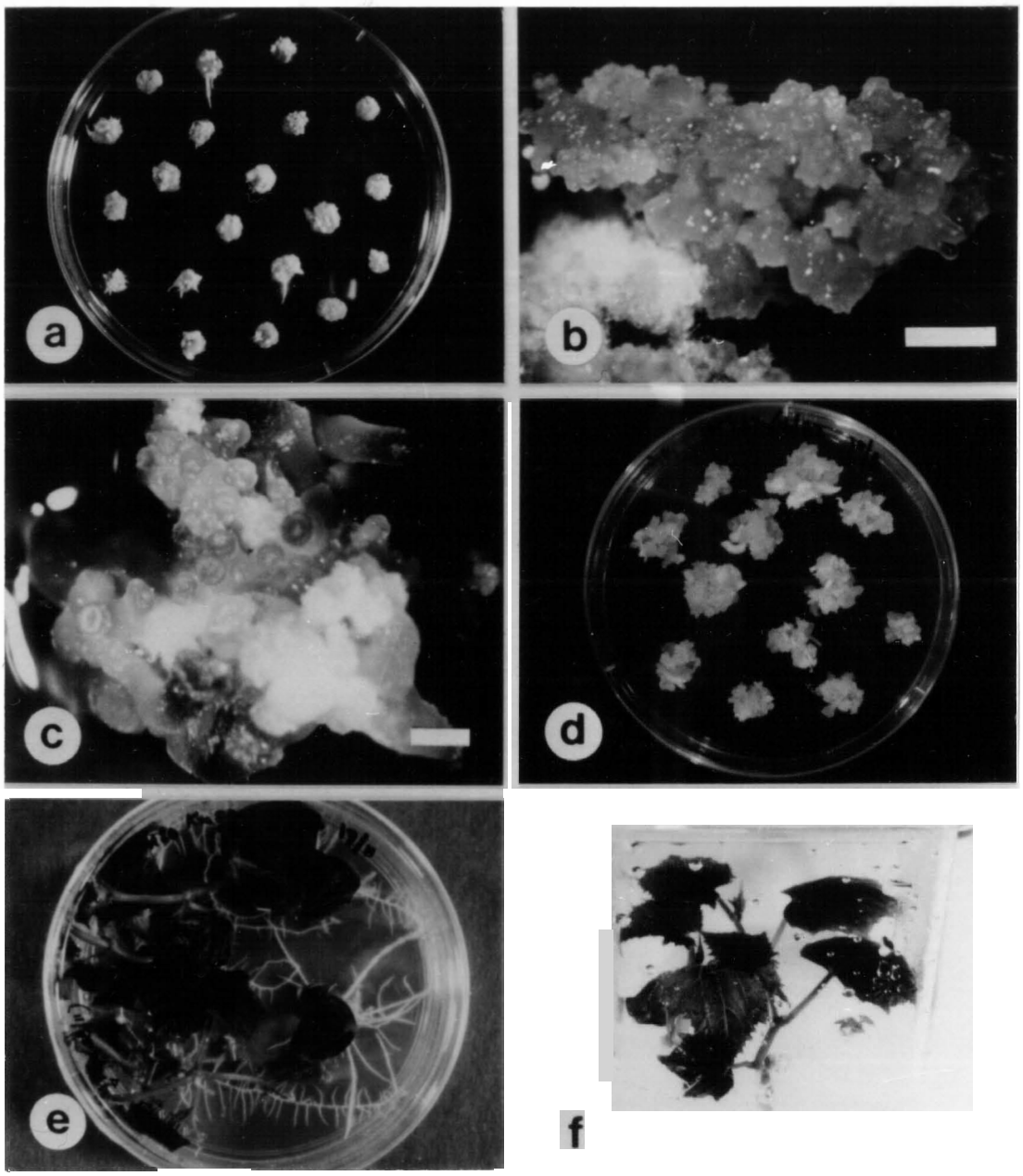
Southern hybridization following Hind-III digestion produced a single band for DNA from plants transformed using EHA105 with pMOG196, two bands for DNA from plantlets transformed with pMOG198, and no band for DNA from a control plant (Fig. 5.4). The bands also indicated the minimal copy number of the transgene in these plants.

Western analysis and chitinase activity assay. Nine plants transformed using pMOG196 (acidic chitinase gene) and two negative controls (untransformed plants) were subjected to Western analysis using an antibody which was raised against the petunia chitinase. The protein, approximately 26 kD, was shown to be present in these nine transformed plants, but not in the two untransformed plants (Fig. 5.5 a). Western analysis using protein extracted from callus and leaf tissue also showed that plants transformed using pMOG198 and pGA492-CHN expressed the

expected tobacco (Fig. 5.5 b) and bean (Fig. 5.5 c) chitinase proteins, respectively.

Based on the measurement of the chitinase activity in the transgenic plants expressing the petunia acidic chitinase gene and in control plants, using a colorimetric assay, there was some variation among four of the transgenic plants (Fig. 5.6). However, plants #2 and #14 shown comparatively higher levels of activity when compared with the untransformed control. The average chitinase activity ratio of transgenic/ control plants was 2.01.

Figure 5.1. Development of transgenic cucumber cv Endeavor plants from cocultivated petiole explants. **a)** Calli developing from petiole explants on MS medium with 2,4-D/BA (5.0/5.0 μM), kanamycin (50 $mg \cdot l^{-1}$) and carbenicillin (500 $mg \cdot l^{-1}$) (selective medium), 4 weeks after cocultivation with strain EHA105/pMOG196; **b)** Embryogenic calli development after several subcultures onto fresh selective medium 12 weeks after cocultivation; **c)** and **d)** Shoot formation from embryogenic cell suspensions plated onto solid MS medium containing 2,4-D/BA (1.0/1.0 μM) or NAA/BA (1.0/1.0 μM) and kanamycin (50 $mg \cdot l^{-1}$); **e)** Plantlets developing roots after two to three subcultures on MS0 medium with kanamycin (50 $mg \cdot l^{-1}$); **e)** Further development of transgenic plantlets after 2 weeks. Bars = 2 mm.


a
b
c
d
e
f

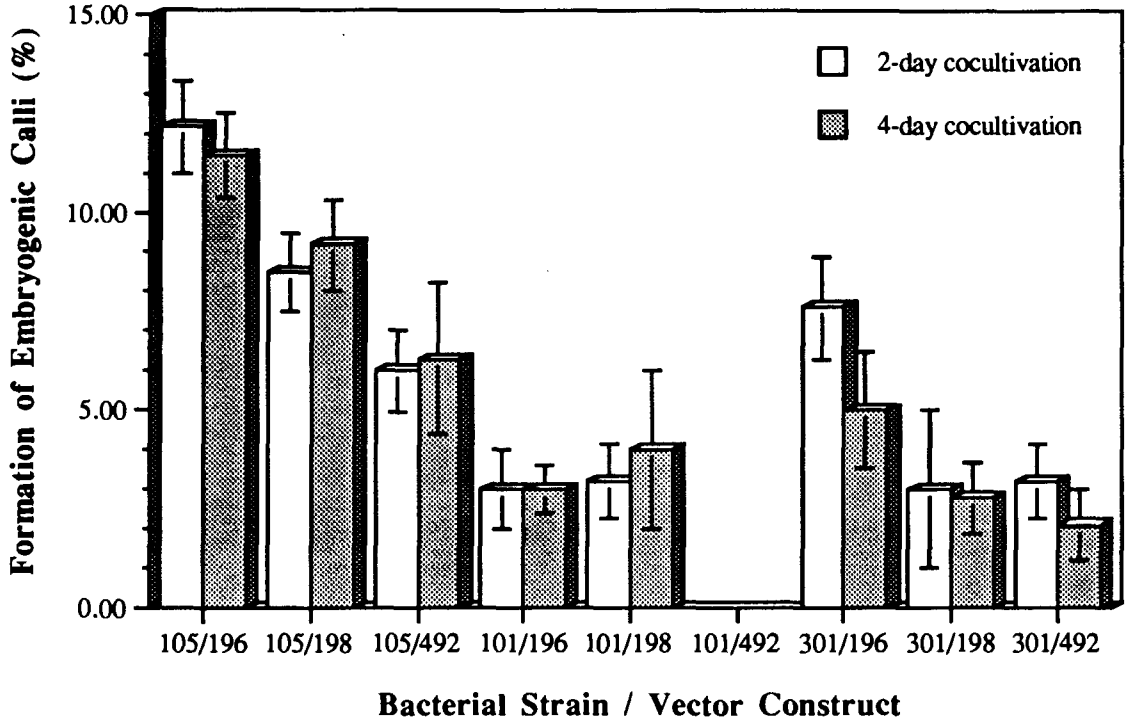


Figure 5.2. Embryogenic callus formation from petiole explants of cucumber cv. Endeavor cocultivated with three *Agrobacterium* strains/vector constructs and for two cocultivation periods. Explants were placed on selection medium containing MS with 2,4-D/BA (5.0/5.0 μM), kanamycin (50 $mg \cdot l^{-1}$) and carbenicillin (500 $mg \cdot l^{-1}$). Data were recorded in week 8 after infection and represent the mean from 12-20 dishes, each with 12 explants. Bars represent standard error of the mean.

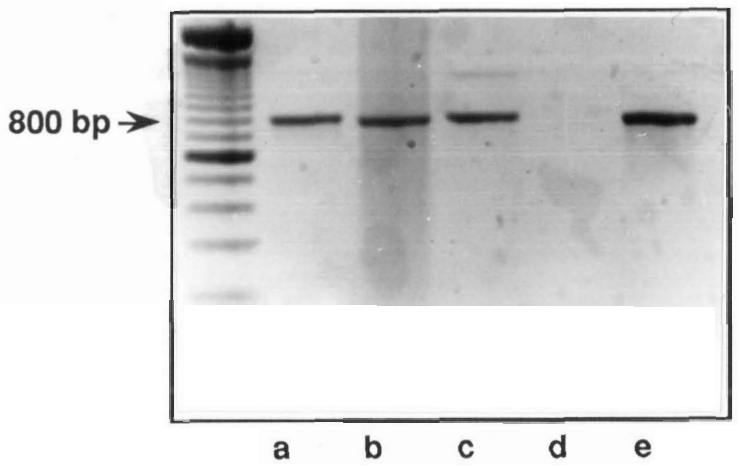


Figure 5.3. PCR amplification of the *NPT II* gene from genomic DNA isolated from transgenic cucumber plants of EHA105/pMOG196 (lane **a**), EHA105/ pMOG198 (lane **b**), EHA105/pGA492-CHN (lane **c**) and negative control (untransformed plant, lane **d**) using two specific primer sequences of the *NPT II* coding region; lane **e** is the positive control (vector DNA).

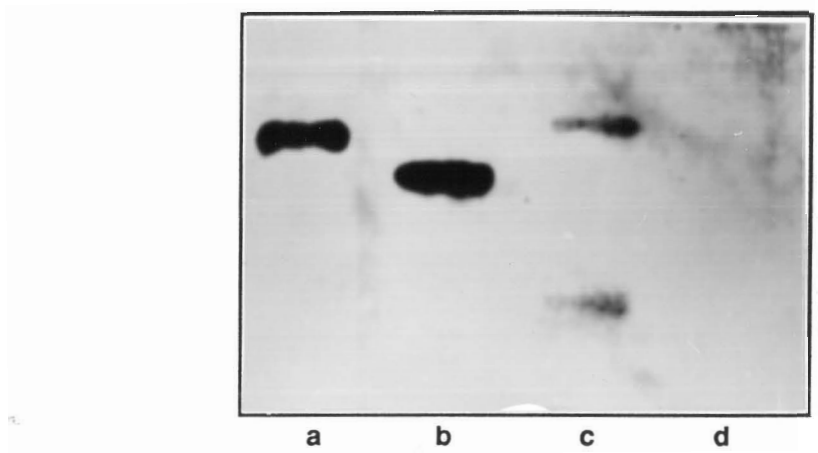
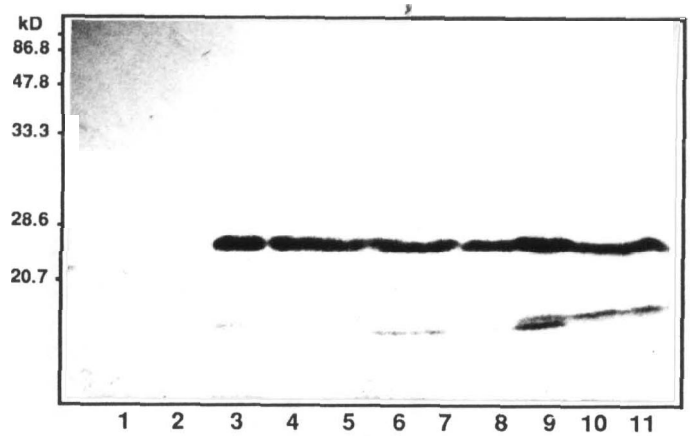
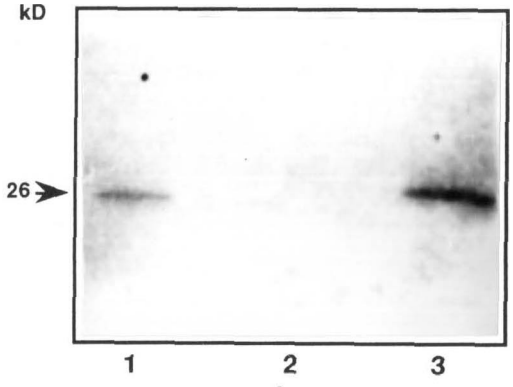


Figure 5.4. Southern blot hybridization of HindIII restricted DNA (10 µg per lane) from transgenic cucumber plants transformed using EHA105/ pMOG196 (lane **b**) and EHA/pMOG198 (lane **c**), and an untransformed (negative) control plant (lane **d**); lane **a** is the positive control (vector DNA). The filter was hybridized against a 800 bp Digoxygenin-UTP labelled fragment containing the *NPT II* coding region. The blot was exposed to X-ray film for 24 h following chemiluminescent detection.

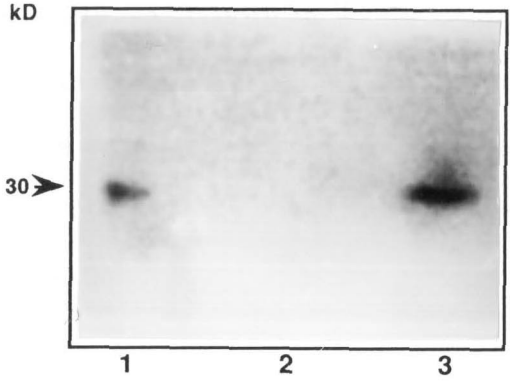
Figure 5.5. Western analysis showing expression of heterologous chitinases in transgenic cucumber plants. **a)** Transgenic plants transformed using pMOG196 with petunia acidic chitinase gene (lanes 3-11); lanes 1-2 are negative controls (untransformed cucumber plants); **b)** Transgenic plant transformed using pMOG198 tobacco basic chitinase gene (lane 3); lane 1 is the positive control (tobacco plant) and lane 2 the negative control (untransformed cucumber plant). **c)** Transgenic plant transformed using pMOG198 tobacco basic chitinase gene (lane 3); lane 1 is the positive control (bean plant) and lane 2 the negative control (untransformed cucumber plant). Samples extracted from leaves were separated by SDS-PAGE, and each of **a)**, **b)** and **c)** probed with an antiserum raised against the respective chitinase. Ten μg protein was loaded per lane.



a



b



c

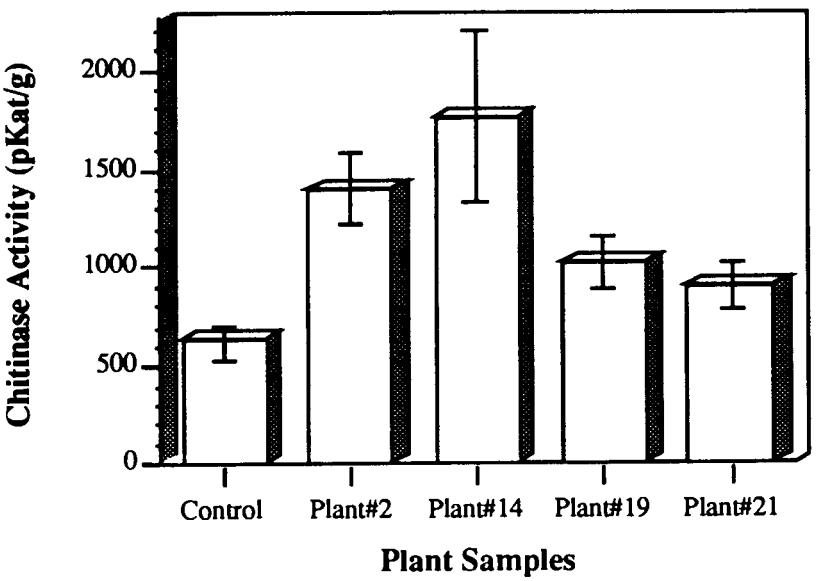


Figure 5.6. Levels of chitinase activity in leaves of different transgenic and control cucumber plants following transformation with pMOG196. One katal (kat) is defined as the enzyme activity catalyzing the formation of 1 mol of N-acetyl-D-glucosamine (or its equivalent) per second. Bars represent standard error of the mean.

5.4 Discussion

This work represents the first report for chitinase gene introduction into pickling cucumber and the expression of the transgene protein in the transgenic plants. Transgenic plants containing chitinase genes have been reported in several other crops (Chapter I), e.g. tobacco, canola (Broglie *et al.*, 1991; Broglie and Broglie, 1993), and tomato (Van den Elzen *et al.*, 1993). The feasibility of transfer of foreign genes into fresh market cucumber has been previously described (Chee, 1990a; Chee and Slightom, 1991).

It was shown in our study that the length of cocultivation, either 2 or 4 d, did not affect the capacity of kanamycin-resistant callus development; nevertheless, calli free of *Agrobacterium* contamination were more difficult to obtain after a 4-d cocultivation. A higher percentage of explants with bacterial ooze was observed after a 4-d cocultivation (unpublished data). This bacterial growth was difficult to eliminate, even after two to three subcultures to fresh selective medium containing 500 $mg \cdot l^{-1}$ carbenicillin.

Direct shoot regeneration from kanamycin-resistant cucumber calli following *Agrobacterium*-mediated transformation has been reported to occur at a low capacity (Chee, 1990a, Sarmento *et al.*, 1992). In this study, shoot or embryo formation from calli derived from cocultivated explants was never observed even after several subcultures onto fresh selective medium over a 3-month period. Therefore, we attempted to multiply the kanamycin-resistant embryogenic aggregates using a suspension culture system (Chapter IV). Suspension cultures proved to be useful for multiplication of embryogenic aggregates and for the production of cell

clumps capable of shoot formation upon transfer onto MS medium devoid of plant growth regulators.

To provide physical evidence for the integration of the foreign DNA into the cucumber genome, PCR amplification of the *NPTII* gene was conducted and yielded the correct size of the band (800 bp) corresponding to the *NPTII* gene in the vectors. In addition, Southern blot analysis of *HindIII* restriction digests of genomic DNA from leaves of the two transgenic lines (each transformed using pMOG196 or pMOG198), which was performed using an *NPT II* probe, yielded a hybridizing signal that corresponded to the inserted fragment(s). Hybridization with the probe revealed one or two bands in the respective transgenic lines, indicating the integration of one or two copies of the *NPT II* gene, respectively. The presence of the heterologous chitinase enzymes was distinguished from background cucumber chitinase levels using antisera corresponding to each chitinase in Western analyses.

Successful gene transfer for crop improvement requires the expression of the introduced genes and production of the foreign protein in transgenic plants. This study showed that heterologous chitinase proteins were produced in transgenic cucumber. The constitutive CaMV *35S* promoter was used and protein extracts from the transgenic plants showed varying but enhanced levels of chitinase activity in leaves compared to the untransformed control. The results from this study demonstrate the feasibility of transforming pickling cucumber with potentially useful agronomic genes.

CHAPTER VI

EVALUATION OF TRANSGENIC CUCUMBER CONTAINING HETEROLOGUOUS CHITINASE GENES FOR RESISTANCE TO FUNGAL PATHOGENS

6.1 Introduction

Higher plants express a group of proteins, the PR proteins, upon infection by pathogenic microorganisms, which are reported to have a role in defence against pathogen development (reviewed by Linthorst, 1990; Bowles, 1992; Stintzi *et al.*, 1993). Among these proteins are a group of hydrolytic enzymes, the chitinases, which have been extensively studied to determine their roles in plants that are challenged by fungal pathogens (reviewed by Punja and Zhang, 1993; Graham and Sticklen, 1994). Inoculation with various pathogens, such as fungi, bacteria and viruses, physical wounding and application of chemical compounds, have all been reported to induce plant chitinases, suggesting that the response may also be stress-induced (Boller *et al.*, 1983; Metraux, 1989; Meins Jr and Ahl, 1989; Rasmussen *et al.*, 1992b; Zhang and Punja, 1994). Two general groups of chitinases are known to occur in plants, i.e. acidic and basic. Acidic (extracellular) chitinases are postulated to attack hyphae of invading fungal pathogens early during pathogenesis and to release fungal cell wall fragments, which in turn may activate other defence-related mechanisms (Mauch and Staehelin, 1989). The basic (vacuolar) forms would affect the

hyphae later upon plant cell collapse and after the cell contents are released into the extracellular space (Keefe *et al.*, 1990)

The expression of genes encoding for antifungal proteins in transgenic plants has been suggested as a strategy to enhance resistance to pathogenic fungi (Nitzsche, 1983; Cornelissen and Melchers, 1993) and to elucidate the role of these proteins in plant defence. Chitinase genes from some plant species and from a bacterium (*S. marcescens*) have been expressed in several transgenic plants, such as tobacco (*Nicotiana tabacum*), *N. sylvestris*, *N. benthamiana*, canola (*Brassica napus*) and tomato (*Lycopersicon esculentum*) (reviewed in Chapter I). In subsequent evaluations of these transgenic plants for increased tolerance to filamentous fungal pathogens, however, the results have not always been conclusive. For example, inoculation of transgenic *N. sylvestris* (Neuhaus *et al.*, 1991b) and *N. benthamina* (Nielsen *et al.*, 1993) with *Cercospora nicotianae* revealed no differences when compared with non-transgenic controls. In contrast, increased chitinase levels in transgenic tobacco (Dunsmuir *et al.*, 1992) and canola (Broglie *at al.*, 1991) significantly reduced susceptibility to *Alternaria longipes* and *R. solani*, respectively.

Cucumber (*C. sativus*) is susceptible to a number of fungal pathogens, some of which are difficult to control through conventional breeding strategies. In an attempt to obtain a broad-spectrum tolerance to these diseases, pickling cucumber cv. Endeavor was genetically engineered to overexpress one of three chitinase genes cloned from different plant species (Chapter V).

The objective of this study was to evaluate these three transgenic lines, each transformed with a chitinase gene originating from petunia (acidic), tobacco (basic) or bean (basic), for response to inoculation with the fungal pathogens *Colletotrichum lagenarium*, *Alternaria cucumerina*, *Botrytis cinerea*, and *Rhizoctonia solani*, which cause anthracnose, leaf spot, grey mould, and stem blight diseases, recpectively (Agrios, 1988).

6.2 Materials and Methods

6.2.1 Plant materials

Transgenic lines of cucumber cv. Endeavor, each containing a chitinase gene from petunia, tobacco (Linthorst *et al.*, 1990) or bean (Broglie *et al.*, 1991), designated 105/196, 105/198 or 105/492, respectively, were evaluated. Numerous transgenic plants of each line were regenerated from transformed embryogenic calli following *A. tumefaciens*-mediated transformation (Chapter V). Non-transgenic plants that were included as controls were obtained as *in vitro*-grown seedlings grown in tissue culture. All plantlets and seedlings at the two true leaf stage were transferred into plastic pots (12.5x12 cm) containing previously-sterilised potting mix (Sunshine Mix 1, Fisons Hort. Inc., Vancouver, BC) in a Conviron growth chamber (with fluorescent lighting, $\approx$40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at plant level) maintained at 24°C, 96% RH, and a photoperiod of 16 $h \cdot d^{-1}$. Plants were evaluated when they were 2 - 6 weeks of age.

6.2.2 Fungal pathogens

The fungal pathogens used in this study were: a) *C. lagenarium* race 1 (culture provided by Dr. J. Kuc, Univ. of Kentucky), used in the evaluation of all three transgenic lines; b) *A. cucumerina* (culture provided by Dr. R.X. Latin, Purdue University), used in the evaluation of transgenic lines expressing tobacco and bean vacuolar chitinases; c) *B. cinerea* (isolated from carrot leaves), used in the evaluation of the transgenic line expressing bean chitinase, and d) *R. solani* (isolated from potato tubers), used in evaluation of the transgenic line expressing bean chitinase. All fungal cultures were maintained on PDA and incubated in the dark at an ambient temperature of 23-25°C. Colonies of *C. lagenarium* were subcultured to fresh medium every 4 - 5 weeks; colonies of the other fungi were subcultured every 2 weeks.

6.2.3 Inoculation procedure

Conidia of *C. lagenarium* were obtained by flooding colonies, 14-20 d after the last subculture, with sterile distilled water and suctioning the spores with a Pasteur pipette. The inoculation procedure used was a modification from the procedure by Dean and Kuc (1986). The spore density was adjusted to 10^7 per ml with the aid of a haemocytometer. Expanded leaves, one leaf per plant, of transgenic and non-transgenic (control) plants were inoculated by applying 16 or 30 5µL-droplets of conidial suspension onto the adaxial surface. For transgenic line 105/196, 10 transgenic and 8 control plants were used; for 105/198, 9 transgenic and 7 control plants were

used; for 105/492, 13 transgenic and 8 control plants were used. To obtain plants of comparable size, transgenic plants were 24-34 d old while control plants were 14-18 d old, after transfer to soil. The lines were evaluated in separate experiments and each experiment was repeated at least once. Transgenic lines 105/198 and 105/492 were also inoculated with *A. cucumerina*. A 12-d-old culture was used as the source of hyphal inoculum. Inoculation was conducted by placing two mycelial plugs (4 mm - diameter) on the adaxial surface of each leaf that was basipetally next to the leaf inoculated with *C. lagenarium*. Both inoculations with *C. lagenarium* and *A. cucumerina* were carried out at the same day.

Transgenic line 105/492 was inoculated with *B. cinerea* and *R. solani* simultaneously. Nine transgenic plants and 12 control plants, 39 and 15 d after transfer to soil, respectively, were used in this test. Cultures of both *B. cinerea* and *R. solani*, 9 d after the last subcultures, were used as the source of hyphal inoculum. Inoculation with *B. cinerea* was conducted by placing two agar plugs (4 mm - diameter) on the adaxial surface of each of the youngest fully expanded leaves of transgenic and control plants, one plug on each side of the leaf. Each leaf that was basipetally next to those inoculated with *B. cinerea* was inoculated with *R. solani*, also by placing two agar plugs of hyphal inoculum. Inoculations with both fungi were carried out at the same day.

Following inoculation, plants were maintained under the same conditions as before inoculation, except that for 24 h after inoculation, the RH within the growth chamber was set at 99%. The development of disease

symptoms was recorded as the number of lesions formed and the diameter of lesions, at times ranging from 3 to 12 d after inoculation. The data for each pathogen were averaged over each experiment, and the standard error of the means for all experiments was calculated.

A detached leaf assay was used to evaluate response to each of *B. cinerea* and *R. solani*. Each leaf was placed, adaxial surface up, in a Petri dish (20x100 mm) containing moistened filter paper. Five leaves of each of 105/492 and control plants were inoculated with *B. cinerea*, by placing two mycelial plugs (4 mm - diameter) on each leaf, one on each side of the leaf. Seven leaves of each of 105/492 and control plants were inoculated with *R. solani*, using the same procedure as for *B. cinerea*. Two control leaves were inoculated with PDA plugs as a negative control. The extent of lesion development was determined after 7-9 d of incubation at 25°C.

6.3 Results

6.3.1 Response to *C. lagenarium* race 1

Leaves inoculated with *C. lagenarium* developed lesions typical of those caused by this pathogen. Disease development was expressed as a percentage of the inoculum droplets which resulted in lesion formation and the average diameter of developing lesions. There was no significant difference in the percentage lesion formation (4 and 12 d post-inoculation) or in the diameter of lesions (12 d post-inoculation) between transgenic 105/196 and control plants (Fig 6.1). Similarly, evaluations of transgenic

lines 105/198 and 105/492 showed that the percentage of lesion formation on transgenic and control plants at all evaluation days (3, 6 and 9 d post-inoculation) was not significantly different (Figs 6.2 a and 6.3 a). However, for the two latest evaluation dates (with lines 105/198 and 105/492), lesions on transgenic plants were slightly larger than those on the control plants (Fig 6.2 b and 6.3 b). All of the results were consistent between leaves on different plants and between experiments.

6.3.2 Response to *B. cinerea* and *R. solani*

Following inoculation of transgenic line 105/492 with *B. cinerea* and *R. solani*, only the former pathogen caused infection as indicated by the formation of visible lesions. Although hyphal growth from inoculum plugs was evident on most of the leaves inoculated with *R. solani*, none or only small irregular lesions were formed; there was no detectable difference in lesion development between transgenic and control plants. With *B. cinerea*, lesion diameters on leaves of transgenic and control plants were 6.7 and 7.0 mm, respectively, on intact plants; lesion sizes were 16.3 and 15.2 mm, respectively, on detached leaves (Figs 6.4 a and b). In all tests, there were no significant differences between the lesion sizes on leaves of transgenic and control plants.

6.3.3 Response to *A. cucumerina*

Following inoculation of transgenic line 105/198 with *A. cucumerina*, hyphae of the fungus grew from inoculum plugs onto the leaf. However, no lesions were visible until 9 d after inoculation, and the

extent of lesion formation was similar on both transgenic and control leaves (data not shown).

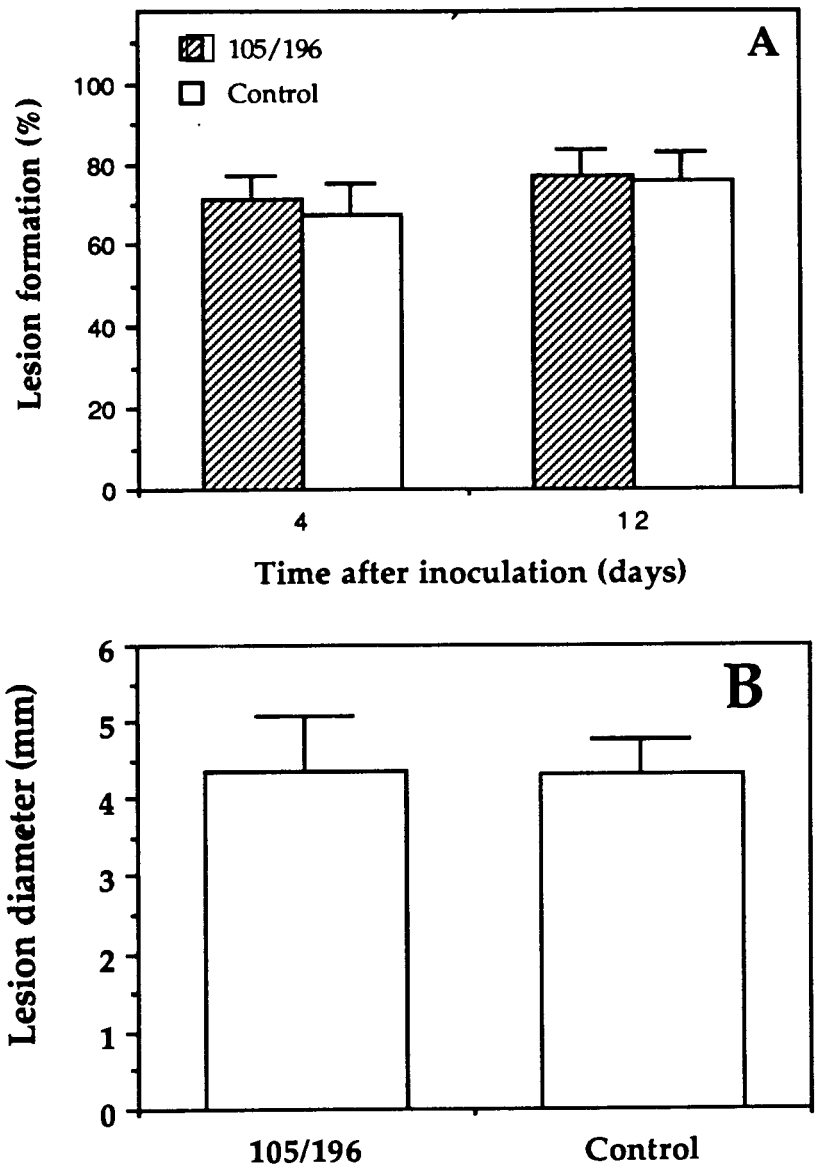


Figure 6.1. Lesion development in cucumber cv. Endeavor transgenic (line 105/196) and control plants following inoculation with *Colletotrichum lagenarium* race 1; **a)** Percentage of lesion formation (4 and 12 days post-inoculation), **b)** Diameter of lesions (12 d post-inoculation). Bars represent standard errors of the mean

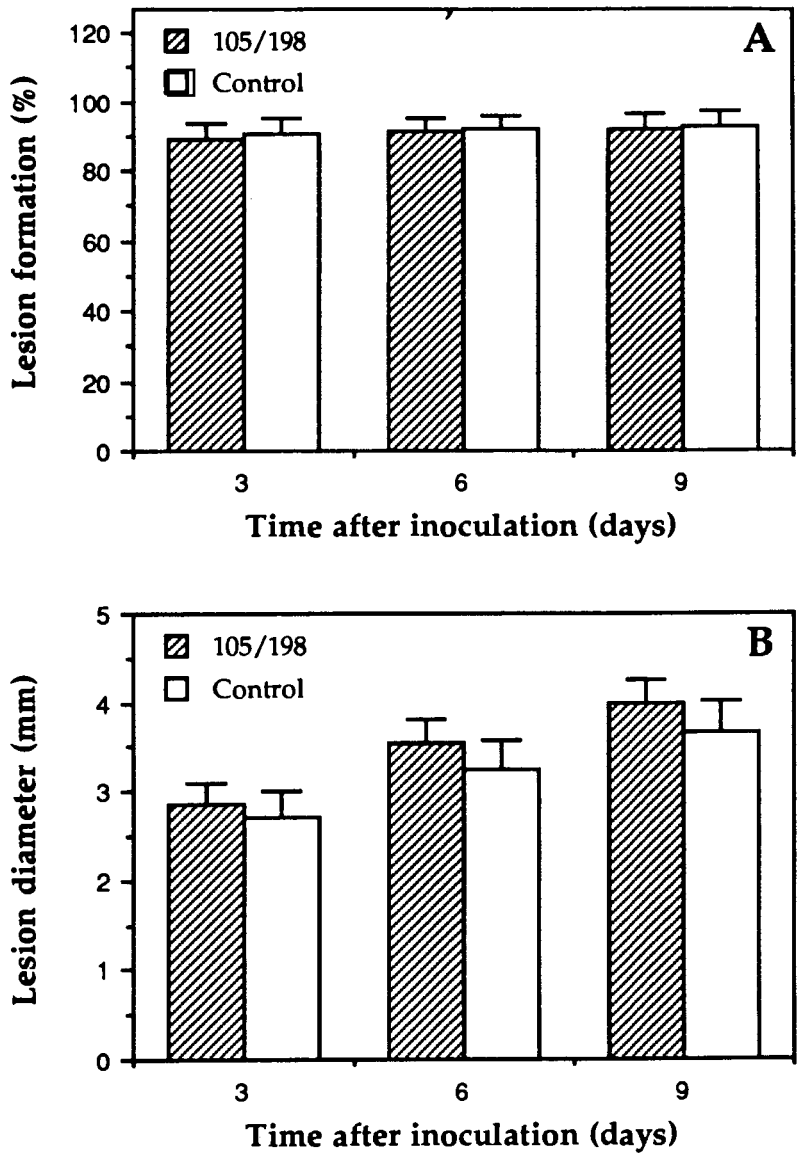


Figure 6.2. Lesion development in cucumber cv. Endeavor transgenic (line 105/198) and control plants following inoculation with *Colletotrichum lagenarium* race 1 (3, 6 and 12 d post-inoculation); **a)** Percentage of lesion formation, **b)** Diameter of lesions. Bars represent standard errors of the mean

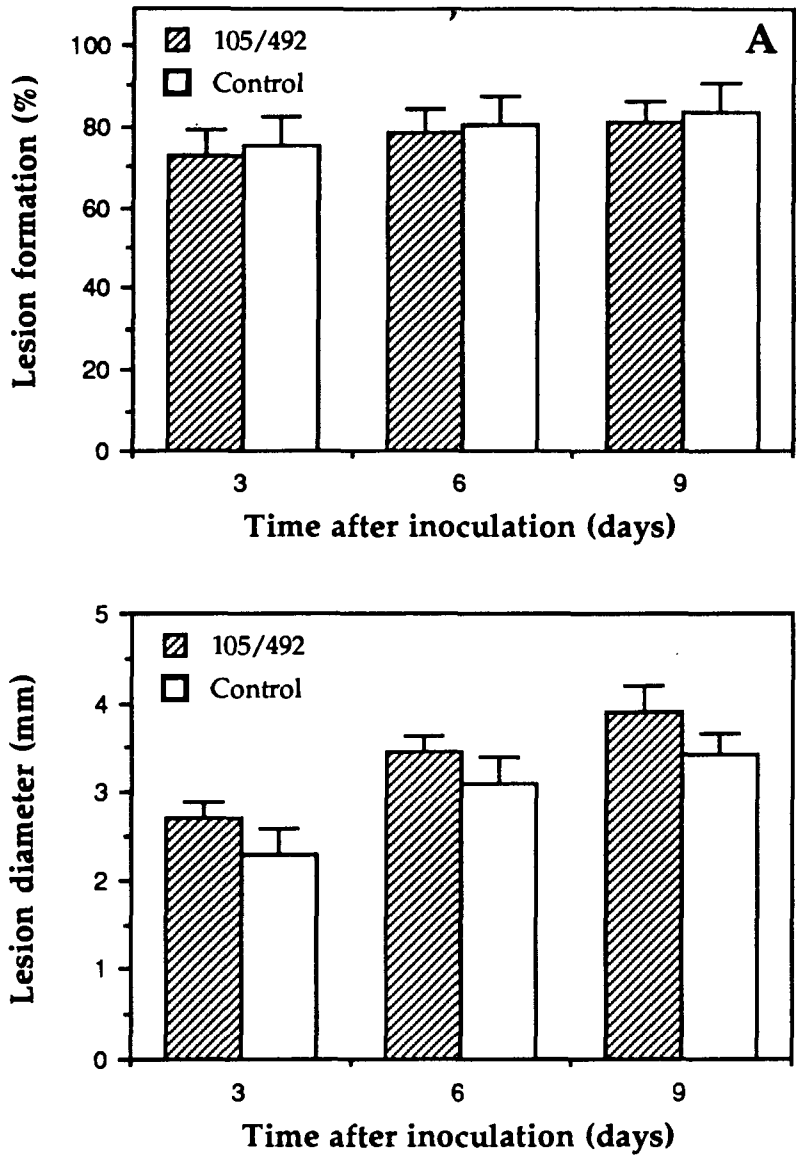


Figure 6.3. Lesion development in cucumber cv. Endeavor transgenic (line 105/492) and control plants following inoculation with *Colletotrichum lagenarium* race 1 (3, 6 and 12 d post-inoculation); **a)** Percentage of lesion formation, **b)** Diameter of lesion. Bars represent standard errors of the mean

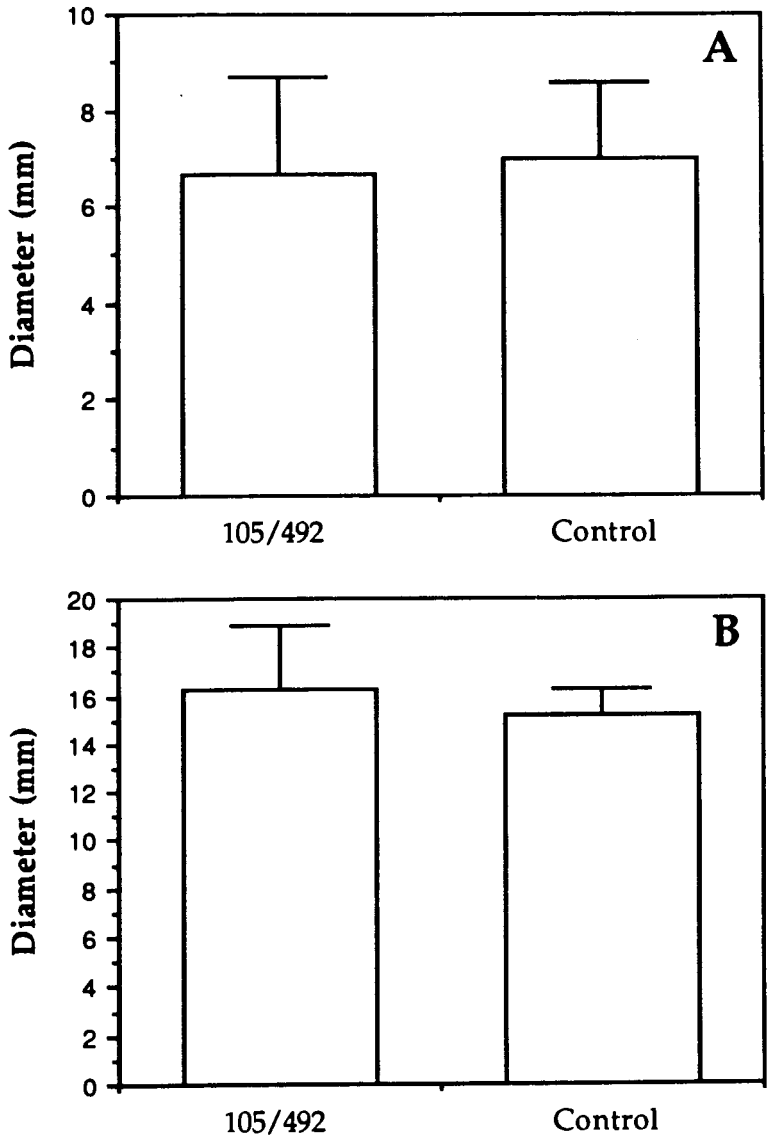


Figure 6.4. Diameter of lesions on leaves of cucumber cv. Endeavor transgenic (line 105/492) and control plants following inoculation with *Botrytis cinerea* (7 d post-inoculation): **a)** Intact plants, **b)** Detached leaves. Bars represent standard errors of mean

6.4 Discussion

The occurrence of chitinases in plants is believed to have a role in defence against filamentous fungal pathogens (Shapira *et al.*, 1989; Sela-Buurlage *et al.*, 1993). Some of the chitinases, either alone or in combination with 1,3-β-glucanases, have been shown to be antifungal *in vitro* (Huynh *et al.*, 1992; Swegle *et al.*, 1992; Melchers *et al.*, 1994) and *in vivo* (in transgenic plants), probably by hydrolysing structural components in the fungal cell wall (Benhamou *et al.*, 1993). The actively growing hyphal tips are most susceptible to lysing upon exposure to chitinases (Graham and Sticklen, 1994).

The vacuolar chitinase from bean has previously been expressed in transgenic tobacco and canola, which resulted in 23- to 44-fold and up to 33-fold increases in chitinase enzyme activity in the leaves, respectively, compared to controls (Broglie *et al.*, 1991; Benhamou *et al.*, 1993). In both species, the transgenic plants showed reduced susceptibility when challenged with *R. solani*. The increased resistance was suggested to be quantitative since the degree of protection observed in the transgenic plants was dependent upon the amount of fungal inoculum used (Broglie *et al.*, 1991).

In another study (Neuhaus *et al.*, 1991b), high expression of a tobacco vacuolar (class I) chitinase in transgenic *N. sylvestris* was reported, in which the transgenic plants accumulated a maximum of 120-fold higher chitinase levels compared to control plants in comparable tissues. In an infection test with *C. nicotianae*, however, no significant increase in resistance was

observed in the transgenic plants, regardless of the fact that the level of chitinase activity in challenged transgenic plants was ca. 19- to 20-fold higher over controls. Class I chitinase used in this study probably was not a limiting factor in protection against *C. nicotianae* (Neuhaus *et al.*, 1991b). Alternatively, during the initial stage of infection, *C. nicotianae* grows intercellularly, and consequently the vacuolar contained chitinase may not affect fungal growth early enough to prevent initial disease development (Punja and Zhang, 1994).

In this study, three transgenic cucumber lines, each expressing a heterologous chitinase from petunia (acidic, extracellular), tobacco (basic, vacuolar), or bean (basic, vacuolar), were evaluated. However, no differences in the response to infection were detected between transgenic and control plants when they were challenged with *C. lagenarium* race 1, *B. cinerea*, and *R. solani*. The lack of an effect could be due to one of several possibilities. Although the chitinase enzymes were produced in the transgenic plants (Chapter V), they may not have increased the overall chitinase expression to a level high enough to result in a significant increase in resistance. The quantitative estimates showed that the increase was 2-fold which is relatively low compared to previous reports in other plant species (Broglie *et al.*, 1991; Neuhaus *et al.*, 1991b). In addition, the susceptibility of *C. lagenarium* race 1 and *B. cinerea* to the two vacuolar chitinases is not known. Fungal cell wall compositions differ depending on the taxonomic group, and fungal species would differ in their susceptibility to chitinases. Therefore, the susceptibility of a pathogen to a certain type of chitinase should be determined in addition to evaluating its effect on

transgenic plants. It has been previously reported that the acidic petunia chitinase (105/196) has little to no antifungal activity (Melchers *et al.*, 1994). Therefore, the lack of an effect in the transgenic line expressing this chitinase was not unexpected.

It has been shown by recent studies that the efficiency of chitinase in transgenic plants can be enhanced by coexpression with glucanases (Van den Elzen *et al.*, 1993; Zhu *et al.*, 1994; Jach *et al.*, 1995). In a study by Zhu *et al.* (1994), transgenic tobacco plants containing either a rice basic chitinase (RCH10) gene or alfalfa acidic glucanase (AGLU1) gene showed only a slight reduction in susceptibility to *C. nicotianae* compared to control controls. Transgenic plants containing both homozygous genes for RCH10 and AGLU1, generated by crossing and selfing the two types of transgenic plants, however, showed significantly high resistance and resulted in ca. 90% reduction in disease development when tested *in planta* using *C. nicotianae*. Therefore, chitinase alone appears to be less effective than when used in conjunction with other enzymes. Additional strategies which are currently being pursued to enhance resistance to fungal pathogens in transgenic plants include the expression of various proteins, such as polygalacturonase-inhibiting proteins (PGIP) (Powell *et al.*, 1994), ribosome-inactivating proteins (RIP) (Jach *et al.*, 1995), and other proteins shown to be antifungal, such as hevein, lectin and thionin (reviewed in Cornelissen and Melchers, 1993; Kamoun and Kado, 1993). The potential of these approaches for enhancing resistance against fungal pathogens will depend on the plant species, the pathogen, and the interaction of the pathogen and the engineered proteins.

REFERENCES

Agrios, G. 1988. Plant Pathology, 3rd ed. Academic Press, New York. 803 p.

An, G. 1987. Binary vectors for plant transformation and promoter analysis. Methods in Enzymol. 153: 292-305.

Anderson, A.R. and L.W. Moore. 1979. Host specificity of the genus *Agrobacterium*. Phytopathol. 69: 320-323.

Baszczyk-Guzek, A. and M. Szwacka. 1994. Selection and characterization of salt-tolerance cucumber (*Cucumis sativus* L.) plants using in vitro culture. Cucurbit Genet. Coop. Rept. 17:40-49.

Benhamou, N., K. Broglie, R. Broglie and I. Chet. 1993. Antifungal effect of bean endochitinase on *Rhizoctonia solani*: Ultrastructural changes and cytochemical aspects of chitin breakdown. Can. J. Microbiol. 39:318-328.

Benzioni, A., S. Mendlinger and M. Ventura. 1991. Effect of sowing dates, temperatures on germination, flowering, and yield of *Cucumis metuliferus*. HortSci. 26:1051-1053.

Bergervoet, J.H.W. and J.B.M. Custers. 1986. Direct and indirect regeneration from cucumber (*Cucumis sativus* L.) explants (Abstr.) Acta Botanica Neerlander 45.

Bergervoet, J.H.W., F. Van der Mark and J.B.M. Custers. 1989. Organogenesis versus embryogenesis from long-term suspension cultures of cucumber (*Cucumis sativus* L.). Plant Cell Rept. 8:116-119.

Beyer Jr, E.M. 1976. A potent inhibitor of ethylene action in plants. Plant Physiol. 58: 268-271.

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

Boller, T. 1988. Ethylene and regulation of antifungal hydrolases in plants. Oxford Surveys Plant Mol. Cell Biol. 5:145-175.

Boller, T. and U. Vogeli. 1984. Vacuolar localization of ethylene-induced chitinase in bean leaves. Plant Physiol. 74: 442-444.

Boller, T., A. Gehri, F. Mauch and U. Vogeli. 1983. Chitinase in bean leaves: induction by ethylene, purification, properties, and possible function. Planta 157:22-31.

Bowles, D.J. 1990. Defense-related proteins in higher plants. Annu. Rev. Biochem. 59:873-907.

Broglie R. and K. Broglie. 1993. Chitinase gene expression in transgenic plants: a molecular approach to understanding plant defence responses. Phil. Trans. R. Soc. (London). B342:265-270.

Broglie, K.E., J.J. Gaynor and R.M. Brogie. 1986. Ethylene-regulated gene expression: Molecular cloning of the genes encoding an endochitinase from *Phaseolus vulgaris*. Proc. Nat. Acad. Sci. USA 83:6820-6824.

Broglie, K.E., P. Biddle, R. Cressman and R. Broglie. 1989. Functional analysis of DNA sequences responsible for ethylene regulation of a bean chitinase gene in transgenic tobacco. Plant Cell 1: 599-607.

Broglie, K., I. Chet, M. Holliday, R. Cressman, P. Biddle, S. Knowlton, C.J. Mauvais and R. Broglie. 1991. Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. Science (Washington D C) 254: 1194-1197.

Cade, R., T.C. Wehner and F.A. Blazich. 1990a. Effect of explant age and growth regulator concentration on adventitious shoot formation from cucumber cotyledonary tissue. Cucurbit Genet. Coop. Rept. 13:14-17.

Cade, R.M., T.C. Wehner and F.A. Blazich. 1990b. Somatic embryos derived from cotyledons of cucumber. J. Amer. Soc. Hort. Sci. 115:691-696.

Chee, P. 1990a. Transformation of *Cucumis sativus* tissue by *Agrobacterium tumefaciens* and the regeneration of transformed plants. Plants Cell Rept. 9:245-248.

Chee, P.P. 1990b. High frequency of somatic embryogenesis and recovery of fertile cucumber plants. HortSci. 52:792-793.

Chee, P.P. 1991a. Plant regeneration from cotyledons of *Cucumis melo* 'Topmark'. HortSci. 26:908-910.

Chee, P.P. 1991b. Somatic embryogenesis and plant regeneration of squash *Cucurbita pepo* L. cultivar YC 60. Plant Cell Rept. 9:620-622.

Chee, P.P. and J.L. Slightom. 1991. Trannsfer and expression of cucumber mosaic virus protein gene in the genome of *Cucumis sativus*. J. Amer. Soc. Hort. Sci. 116:1098-1102.

Chee, P.P. and D.M. Tricoli. 1988. Somatic embryogenesis and plants regeneration from cell suspension cultures of *Cucumis sativus* L. Plants Cell Rept. 7:274-277.

Colijn-Hooymans, C.M. and J.H.C. Hakkert. 1991. Transformation and regeneration of different cucumber cultivars (Abstr. no. 466). In vitro: Cell. Dev. Biol. 27: 153a

Collinge, D.B., K.M. Kragh, J.D. Mikkelsen, K.K. Nielsen, U. Rasmussen and K. Vad. 1993. Plant chitinases. Plant J. 3:31-40.

Cornelissen, J.C. and L.S. Melchers. 1993. Strategies for control of fungal diseases with transgenic plants. Plant Physiol. 101: 709-712.

Custers, J.B.M. and J.H.W. Bergervoet. 1980. In vitro adventitious bud formation on seedlling and embryo explants of *Cucumis sativus* L. Cucurbit Genet. Coop. Rept. 3:2-4.

Custers, J.B.M. and E.C.P. Verstappen. 1989. Improvement of in vitro growth of cucumber. Cucurbit Genet. Coop. Rept. 12:20-22.

Custers, J.B.M., J.E.M. Van Deelen and J.H.W. Bergervoet. 1988. Development of callus and somatic embryos fron zygotic embryos of cucumber (*Cucumis sativus* L.). Cucurbit Genet. Coop. Rept. 11:1-2.

Dabauza, M, L.A. Roigand and V. Moreno. 1991. Selective methods for the recovery of somatic hybrids of *Cucumis melo* X *C. metuliferus* and *C. sativus* X *C. metuliferus*. Cucurbit Genet. Coop. Rept. 14:81-84.

Davis, J.M., H.R.G. Clarke, H.D.J. Barnshaw and M.P. Gordon. 1991. *Populus* chitinase genes: Structure, organization and similarity of translated sequences to herbaceous plant chitinases. Plant Mol. Biol. 17:631-640.

Dean, R.A. and J. Kuc. 1986. Induced systemic protection in cucumber: Effect of inoculum density on symptom development caused by *Colletotrichum lagenarium* in previously infected and uninfected plants. Phytopathol. 76:186-189.

De Block, M. 1988. Genotype-independent leaf disc transformation of potato (*Solanum tuberosum*) using *Agrobacterium tumefaciens*. Theor. Appl. Genet. 76:767-774.

De Ponti, O.M.B. 1980. International Course on Plant Breeding. Breeding of Cucumber, International Agricultural Centre, Wageningen, The Netherlands. 13 p.

Deakin, J.R., G.W. Bohn and T.W. Whitaker. 1971. Interspecific hybridization in *Cucumis*. Econ. Bot. 25:195-211.

Donnelly, D.J. and W.E. Vidaver. 1988. Glossary of Plant Tissue Culture,, Vol.3 , Advance in Plant Sciences Series. Dioscorides Press, Portland.

Draper, J. and R. Scott. 1991. Gene transfer to plants, p.38-81. In: D. Grierson (ed.) Plant Genetic Engineering, Vol.1, Chapman and Hall, New York.

Draper, J., R. Scott, A. Armitage and R. Walden. 1988. Plant Genetic Transformation and Gene Expression. A Laboratory Manual. Blackwell Sci. Publ., Oxford.

Dunsmuir, P. and T. Suslow. 1989. Structure and regulation of organ- and tissue-specific genes: Chitinase genes in plants, p.215-227. In: I.K. Vasil (ed.) Cell Culture and Somatic Cell Genetic of Plants, Vol.6, Academic Press, New York.

Dunsmuir, P., W. Howie, E. Newbigin. L. Joe, E.Penzes and T. Suslow. 1992. Resistance to *Rhizoctonia solani* in transgenic tobacco, p. 5676-571. In: W. Nester and D.P.S. Verma (eds.) Advences in Molecular Genetics of Plant-Microbe Interactions, vol 2. Kluwer Acad. Publ., Dordrecht.

Esquinas-Alcazar, J.T. and P.J. Gilick. 1983. Genetic Resources of Cucurbitaceae. International Board for Plant Genetic Resources Bulletin, FAO/UN, Rome, Italy. 101 p.

Fassuliotis, G. 1967. Species of *Cucumis* resistant to the root-knot nematode *Meloidogyne incognita acrita*. Plant Dis. Reporter 51:720-723.

Finer, J.J. and M.D. McMullen. 1990. Transformation of cotton (*Gossypium hirsutum* L.) via particle bombardment. Plant Cell Rept. 8:586-589.

Gambley, R.L. and W.A. Dodd. 1990. An in vitro technique for production de novo of multiple shoots in cotyledon explants of cucumber (*Cucumis sativus* L.). Plant Cell Tis. Org. Cult. 20:177-183.

Gordon-Kamm, W.J., T.M. Spencer, M.L. Mangano,T.R. Adams, R.J. Daines, W.G. Star, J.V. O'Brie, C.A. Chambers, W.R. Adams, Jr., N.G. Willetts, T.B. Rice, C.J. Mackey, R.W. Krueger, A.P. Kausc and P.J. Lemaoux. 1990. Transformation of maize cells and regeneration fertile transgenic plants. Plant Cell 2: 603-618.

Gheysen, G., G. Angenon and M. Van Motagu. 1992. Transgenic plants: *Agrobacterium*-mediated transformation and its use for crop improvement, In: J.A.H. Murray (ed.) Transgenesis. Application of Gene Transfer, John Wiley and Sons, Chichester.

Graham, L.S. and M. Sticklen. 1994. Plant chitinases. Can. J. Bot. 72: 1057-1083.

Hammar, S.A. and R. Grumet. 1990. Regeneration and *Agrobacterium tumefaciens*-mediated transformation of cucumber (*Cucumis sativus* L.) (Abstr.). HortSci. 25:70.

Handley, L.W. and O.L. Chambliss. 1979. In vitro propagation of *Cucumis sativus* L. HortSci. 14:22-23.

Hernandez, M.O. 1994. Tissue culture and *Agrobacterium*-mediated transformation of carrot (*Daucus carota* L.). MSc. Thesis. The Department of Biological Sciences, Simon Fraser University, British Columbia, Canada. 79 p.

Hoekema, A., P.R. Hirsch, P.J.J. Hooykaas and R.A. Schilperoort. 1983. A binary plant vector strategy based on separation of vir and T-region of the *A. tumefaciens* Ti-plasmid. Nature 303:179-181.

Hooykaas, P.J.J. and R.A. Schilperoort. 1992. *Agrobacterium* and plant genetic engineering. Plant Mol. Biol. 19:15-38.

Horsch, R.B., J.E. Fry, N.L. Hoffmann, D. Eichholtz, S.G. Rogers and R.T. Fraley. 1985. A simple and general method for transferring genes into plants. Science 227: 1229-1231.

Horsch, R.B., J. Fry, M.A.W. Hinchee, H.J. Klee, S.G. Rogers and R.T. Fraley. 1988. Strategies for practical gene transfer into agronomically inportant crops, p.13-19. In: R.T. Fraley, N.M. Frey and J. Schell (eds.) Current Communications in Molecular Biology. Genetic improvements of Agriculturally Important Crops. Progress and Issues, Cold Spring Harbor Laboratory, New York.

Howie, W., L. Joe, E. Newbigin, T. Suslow and P. Dunsmuir. 1994. Transgenic tobacco plants which express the *chiA* gene from *Serratia marcescens* have enhanced tolerance to *Rhizoctonia solani*. Transgenic Research 3:90-98.

Huynh, Q.K., C.M. Hironaka, E.B. Levine, C.E. Smith, J.R. Borgmeyer and D.M. Shah. 1992. Antifungal proteins from plants: Purification, molecular cloning, and antifungal properties of chitinases from maize seed. J. Biol. Chem. 267:6635-6640.

Jach, G., B. Gornhardt, J. Mundy, J. Logemann, E. Pinsdorf, R. Leah, J. Schell and C. Mass. 1995. Enhanced quantitative resistance against fungal disease by combinatorial exprassion of different barley antifungal proteins in transgenic tobacco. The Plant J. 8:97-109.

Jelaska, S. 1972. Embryoid formation by fragments of cotyledons and hypocotyls in *Cucurbita pepo*. Planta 103:278-280.

Jones, J.D.G., C. Dean, D. Gidoni, D. Gilbert, D. Bond-Nutter, R. Lee, J. Bedbrook and P. Dunsmuir. 1988. Expression of bacterial chitinase protein in tobacco leaves using two photosynthetic promoter. Mol. Gen. Genet. 212:536-542.

Kamoun, S. and C.I. Kado. 1993. Genetic engineering for plant disease resistance, p.165-183. In: L. Kim (ed.) Advanced Engineered Pesticides, Marcel Dekker, New York.

Keefe, D., U. Hinz and F.M. Jr. 1990. The effect of ethylene on the cell-type specific and intracellular localization of β-1,3-glucanase and chitinase in tobacco leaves. Planta 182:43-51.

Kho, Y.O., A.P.M.D. Nijs and J. Franken. 1980. Interspecific hybridization in *Cucumis* L. II. The crossability of species, an investigation of *in vivo* pollen tube growth and seed set. Euphytica 29:661-672.

Kim, S-G., J-R. Chang, H.C. Cha and K.W. Lee. 1988. Callus growth and plant regeneration in diverse cultivars of cucumber (*Cucumis sativus* L.). Plant Cell Tis. Org. Cult. 12:67-74.

Kim, Y-H. and J. Janick. 1989. Somatic embryogenesis and organogenesis in cucumber. HortSci. 24:702.

Klee, H., R. Horsch and S. Rogers. 1987. *Agrobacterium*-mediated plant transformation and its further applications to plant biology. Ann. Rev. Plant Physiol. 38:467-486.

Komari, T. 1989. Transformation of cultures of nine plant species mediated by *Agrobacterium*. Plant Sci. 60:223-229.

Kombrink, E., M. Schroder and K. Hahlbrock. 1988. Several "pathogenesis-related" proteins in potato are 1,3-β-glucanases and chitinases. Proc. Nat. Acad. Sci. USA 85:782-786.

Kragh, K.M., S. Jacobsen, J.D. Mikkelsen and K.A. Nielsen. 1991. Purification and characterization of three chitinases and one beta-1,3-glucanase accumulating in the medium cell suspension cultures of barley (*Hordeum vulgare* L.). Plant Sci. (Limerick) 76:65-78.

Kurosaki, F., N. Tashiro and A. Nishi. 1988. Role of chitinase and chitin oligosaccarides in lignification response of cultured carrot cells treated with mycelial walls. Plant Cell Physiol. 29:527-531.

Ladyman, J.A.R. and J. Forsman. 1991. Transformation of cucumber and observations on regenerated seedings - with particular attention to localized GUS activity on non-transformed plants (Abstr). Suppl. to Plant Physiol. 96:95.

Lawton, K., E. Ward, G. Payne, M. Moyer and J. Ryals. 1992. Acidic and basic class III chitinase mRNA accumulation in response to TMV infection of tobacco. Plant Mol. Biol. 19:735-743.

Lazarte, J.E. and C.C. Sasser. 1982. Asexual embryogenesis and planlet development in anther culture of *Cucumis sativus* L. HortSci. 17:88.

Legrand, M., S. Kauffmann, P. Geoffrey and B. Fritig. 1987. Biological function of pathogenesis-related proteins: Four tobacco pathogenesis-related proteins are chitinases. Proc. Nat. Acad. Sci. USA 84: 6750-6754.

Lentini, E., H. Mussel, M.A. Mutschles and E.D. Earle. 1989. Ethylene generation and reversal of ethylene effects during development in vitro of rapid-cycling *Brassica campestris*. Plant Sci. 54:75-81.

Lichtenstein, C. and J. Draper. 1985. Genętic engineering of plants, p.67-119. In: D.M. Glover (ed.) DNA Cloning: A Practical Approach, Vol.3, IRL Press, Oxford, Washington DC.

Linthorst, H.J.M., L.C. Van Loon, C.M.A. Van Rossum, A. Mayer, J.F. Bol, J.S.C. Van Roekel, E.J.S. Meulenhoff and B.J. Cornelissen. 1990. Analysis of acidic and basic chitinases from tobacco and petunia and their constitutive expression in transgenic tobacco. Mol. Plant-Microbe Interactions 3:252-258.

Lou, H. and S. Kako. 1994. Somatic embryogenesis and plant regeneration in cucumber. HortSci. 29:906-909.

Lower, R.D. and M.D. Edwards. 1986. Cucumber breeding, p.173-207. In: M.J. Bassett (ed.) Breeding Vegetable Crops, AVI Pub. Co., Westport, Conn. 584 p.

Mackay, W.A., T.J. Ng and F.A. Hammerschlag. 1994. *Cucumis melo* L. callus response to toxins produced by *Myrothecium roridum* Tode ex. Fries. J. Amer. Soc. Hort. Sci. 119:356-360.

Majeau, N., J. Trudel and A. Asselin. 1990. Diversity of cucumber chitinase isoforms and characterizations of one seed basic chitinase with lysozyme activity. Plant Sci. 68:9-16.

Malepszy, S. 1988. Cucumber (*Cucumis sativus* L.), p.277-293. In: Y.P.S. Bajaj (ed.) Biotechnology in Agriculture and Forestry, Vol.6, Springer and Verlag, Berlin and Heidelberg.

Malepszy, S. and El-Kazzaz. 1990. In vitro culture of *Cucumis sativus*. XI. Selection of resistance to *Fusarium oxysporium*. Acta Hort. 280:455-458.

Malepszy, S. and A. Nadolska-Orczyk. 1983. In vitro culture of *Cucumis sativus*. I. Regeneration of plantlets from callus formed by leaf explants. Z. Pflanzenphysiol. 111:273-276.

Malepszy, S. and E. Solarek. 1986. In vitro culture of *Cucumis sativus* L. IV. Conditions for cell suspension. Genetica Polonica 27:249-253.

Marton, L. and J. Browse. 1991. Facile transformation of *Arabidopsis*. Plant Cell Rept. 10: 235-239.

Mathews, H. 1988. In vitro Responses of *Brassica juncea* and *Vigna radiata* to the antibiotic kanamycin. Ann. Bot. 62: 671-675.

Mathews, H., R.E. Litz, H.D. Wilde, S.A. Merkle and H.Y. Wetzstein. 1992. Stable integration and expression of β-glucuronidase and NPT II genes in mango somatic embryos. In Vitro Cell Dev. Biol. 28P:172-178.

Mauch, F. and L.A. Staehelin. 1989. , Functional implication of the subcellular localization of ethylene-induced chitinase and β-1,3-glucanase in bean leaves. Plant Cell 1:447-457.

Mauch, F., B. Mauch-Máni and T. Boller. 1988. Antifungal hydrolases in pea tissue: II. Inhibition of fungal growth by combinations of chitinase and β-1,3-glucanase. Plant Physiol. 88: 836-942.

McCarthy, W.H., T.C. Wehner and M.E. Daub. 1989. Isolation and culture of *Cucumis metuliferus* protoplasts. Cucurbit Genet. Coop. Rept. 12:26-29.

McInnes, E., A.J. Morgan, B.J. Mulligan and M.R. Davey. 1991. Roots induced on cucumber cotyledons by the agropine Ri plasmid TR-DNA exhibit the transformed phenotype. Plant Cell Rept. 9: 647-650.

McMullen, M.D. and J.J. Finer. 1991. Stable transformation of cotton and soybean embryogenic culture via microprojectile bombardment J. Cell Biochem. 14E:285.

Meins Jr, F. and P. Ahl. 1989. Induction of chitinase and β-1,3-glucanase in tobacco plants infected with *Pseudomonas tabaci* and *Phytophthora parasitica* var *nicotianae*. Plant Sci. 61:155-161.

Meins Jr, F., B. Fritig, H.J.M. Linthorst, J.D. Mikkelsen, J.-M. Neuhaus and J. Ryals. 1994. Plant chitinase genes. Plant Mol. Biol. Reporter Suppl. to Vol. 12: S22-S28.

Melchers, L.S. and P.J.J. Hooykaas. 1987. Virulence of *Agrobacterium*. Oxford Surveys of Plant Mol. Cell Biol. 4:167-220.

Melchers, L.S., M.B. Sela-Buurlage, S.A. Vloemans, C.P. Woloshuk, J.S.C. Van Roekel, J. Pen, P.J.M. Van den Elzen and B.J.C. Cornelissen. 1993. Extracellular targeting of the vacuolar tobacco proteins AP24, chitinase and β-1,3-glucanases in transgenic plants. Plant Mol. Biol. 21: 583-93.

Metraux, J.P. 1986. Local and systemic induction of chitinase in cucumber plants in response to viral, bacterial and fungal infections. Physiol. Mol. Plant Pathol. 28:161-169.

Metraux, J.P., W. Burkhart, M. Moyer, S. Dicher, W. Middlestead, S. Williams, G. Payne, M. Carnes and J. Ryals. 1989. Isolation of a complementary DNA encoding a chitinase with structural homology to a bifunctional lysozyme/chitinase. Proc. Nat. Acad. Sci. USA 86:896-900.

Mettler, I.J. 1987. A simple and rapid method of DNA from tissue cultured plant cells. Plant Mol. Biol. Reporter 5: 346-349.

Miki, B.L.A. and V.N. Iyer. 1990. Fundamental of gene transfer in plants, p.473-489. In: D.T. Dennis and D.H. Turpin (eds.) Plant Physiology, Biochemistry and Molecular Biology, Longman Scientific and Technical, Essex, England.

Moreno, V. and L.A. Roig. 1990. Somaclonal variation in cucurbits, p.435-464. In: Y.P.S. Bajaj (ed.) Biotechnology in Agriculture and Forestry, Vol.11, Somaclocal Variation in Crop Improvement 1. Springer-Verlag, Berlin Heidelberg.

Msikita, W., H.T. Wilkinson and R.M. Skirvin. 1990. Resistance of in vitro-derived cucumber plants to *Pythium aphanidermatum*. HortSci. 25:967-969.

Mukhopadhyay, A., N. Arumugam, P.B.A. Nandakumar, A.K. Pradhan, V. Gupta and D. Pental. 1992. *Agrobacterium*-mediated genetic transformation of oilseed *Brassica campestris*: Transformation frequency is strongly influenced by the mode of shoot regeneration. Plant Cell Rept. 11:506-513.

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

Neuhaus, J.M., L. Sticher, F.J. Meins and T. Boller. 1991a. A short C-terminal sequence is necessary and sufficient for the targeting of chitinases to the plant vacuole. Proc. Nat. Acad. Sci. USA 88:10362-10366.

Neuhaus, J.-M., P. Ahl-Goy.. U. Hinz, S. Flores and F. Meins. 1991b. High-level expression of a chitinase gene in *Nicotiana sylvestris*. Susceptibility of transgenic plants to *Cercospora nicotianae* infection. Plant Mol. Biol. 16:141-151.

Nielsen, K.K., J.D. Mikkelsen, K.M. Kragh and K. Bojsen. 1993. An acidic Class III chitinase in sugar beet: Indictuion by *Cercospora beticola*, characetization, and expression in transgenic tobacco plants. Mol. Plant-Microbe Interactions 6: 495-506.

Nishizawa, Y. and T. Hibi. 1991. Rice chitinase gene: cDNA cloning and strees-induced expression. Plant Sci. 76:211-218.

Nitzsche, W. 1983. Chitinase as a possible resistance factor for higher plants. Theor. Appl. Genet. 65:171-172.

Novak, F.J. and M. Dolezelova. 1982. Hormone control of growth and differentiation in the *in vitro* cultured tissue of cucumber (*Cucumis sativus* L.). Biologia (Bratislava) 37: 283-289.

Orczyk W., A. Nadolska-Orczyk and S. Mąlepzy. 1988. Plant regeneration of wild *Cucumis* species. Acta. Societ. Botanic Poloniae 57:195-200.

Palmer, C.E. 1992. Enhanced shoot regeneration from *Brassica campestris* by silver nitrate. Plant Cell Rept. 11:541-545.

Park, Y.G. and S.H. Son. 1988. Regeneration of plantlets fron cell suspension culture derived callus of white poplar (*Populus alba* L.). Plant Cell Rept. 7:567-570.

Park, R.D., K.S. Song and I.W. Jung. 1992. Antifungal activity of a chitinase purified from bean leaves. J. Korean Agric. Chem. Soc. 35:191-195.

Pauk, J., S. Fekete and I. Stefanov. 1991. Protoplast and direct genetic transformation experiments in rape. Novenytermeles 40: 193-202.

Potrykus, I. 1990. Gene transfer to plants: assesment and perspectives. Physiol. Plant. 79: 125-134.

Powell, A.L.T., D. D'hallewin, B.D. Hall, H. Stolz, J.M. Labawitch, A.B. Bennet. 1994. Glycoprotein inhibitors of fungal polygalacturonase: Expression of pear PGIP improves resistance in transgenic tomatoes. Abstr. no. 1904. The Fourth International Congress for Plant Molecular Biology, June 19-22, Amsterdam.

Provvidenti, R. and R.W. Robinson. 1974. Resistance to squash mosaic and watermelon mosaic virus 1 in *Cucumis metuliferus*. Plant Dis. Reporter 58:735-738.

Pua, E.-C. 1990. Somatic embryogenesis and plant regeneration from hypocotyl protoplasts of *Brassica juncea* (L.) Czern & Coss. Plant Sci. 68:231-238.

Punja, Z.K. and S.H.T. Raharjo. 1993. Regeneration of plants from protoplasts of *Cucumis sativus* L. (cucumber), p.31-45. In: Y.P.S. Bajaj (ed.) Biotecnology in Agriculture and Forestry, Vol.22, Plant Protoplasts and Genetic Engineering. Springer-Verlag, Berlin Heidelberg

Punja, Z.K. and Y.Y. Zhang. 1993. Plant chitinases and their roles in resistance to fungal diseases. J. Nematol. 25:526-540.

Punja, Z.K., F.A. Tang and L.H. Watkins. 1988. Identification of resistance to root knot nematodes and virus diseases in *Cucumis metuliferus* and approaches to hybridization with *Cucumis sativus* by protoplast fusion (Abstr.). Phytopathol. 78:1578.

Punja Z.K., F.A. Tang and G.G. Sarmento. 1990a. Isolation, culture and plantlet regeneration from cotyledon and mesophyll protoplasts of two pickling cucumber (*Cucumis sativus* L.) genotypes. Plant Cell Rept. 9:61-64.

Punja, Z.K., N. Abbas, G.G. Sarmento and F.A. Tang. 1990b. Regeneration of *Cucumis sativus* var. *sativus* and *C. sativus* var. *hardwickii*, *C. melo*, and *C. metuliferus* from explants through somatic embryogenesis and organogenesis. Plant Cell Tis. Org. Cult. 21:93-102.

Purnhauser, L., P. Medgyesy, M. Czako, D. P.J. and L. Marton. 1987. Stimulation of shoot regeneration in *Triticum aestivum* and *Nicotiana flambaginifolia* Viv. tissue culture using ethylene inhibitor $AgNO_3$. Plant Cell Rept. 6: 1-4.

Rasmussen, U., K. Bojsen and K.B. Collinge. 1992a. Cloning and characterization of a pathogen-induced chitinase in *Brassica napus*. Plant Mol. Biol. 20:277-287.

Rasmussen, U., H. Giese and J.D. Mikkelsen. 1992b. Induction and purification in chitinase in *Brassica napus* ssp. *oleifera* infected with *Phoma lingam*. Planta (Heidelberg) 187:328-334.

Robinson, R.W. and T.W. Whitaker. 1974. *Cucumis*, p.144-150. In: R.C. King (ed.) Handbook of Genetics, Vol.2, Plants, Plant Viruses, and Protists. Plenum Press, New York and London.

Roby, D., K. Broglie, R. Cressman, P. Biddle, I. Chet and R. Broglie. 1990. Activation of a bean chitinase promoter in transgenic tobacco plants by phytopathogenic fungi. Plant Cell 2: 999-1007.

Rousseau-Limouzin, M. and B. Fritig. 1991. Induction of chitinases, 1,3-β-glucanases and other pathogenesis related proteins in sugar beet leaves upon infection with *Cercospora beticola*. Plant Physiol. Biochem. 29:105-117.

Roustan, J.P., A. Latche and J. Fallot. 1992. Enhancement of shoot regeneration from cotyledons of *Cucumis melo* by silver nitrate O-3, an inhibitor of ethylene action. J. Plant Physiol. 140:485-488.

Samac, D.A. and D.M. Shah. 1991. Developmental and pathogen-induced activation of the *Arabidopsis* acidic chitinase promoter. Plant Cell 3: 1063-1072.

Samac, D., C.M. Hironaka, P.E. Yallaly and D.M. Shah. 1990. Isolation and characterization of the genes encoding basic and acidic chitinase in *Arabidopsis thaliana*. Plant Physiol. 93: 907-914.

Sapountzakis, G. and A.S. Tsaftaris. 1994. Micropropagation of the cucumber hybrids 'Brumex' and 'Bambina'. Cucurbit Genet. Coop. Rept. 17:50-53.

Sarmento, G.G., K. Alpert, F.A. Tang and Z.K. Punja. 1992. Factors influencing *Agrobacterium tumefaciens* mediated transformation and

expression of kanamycin resistance in pickling cucumber. Plant Cell Tis. Org. Cult. 31:185-193.

Schlumbaum, P., F. Mauch, U. Vogeli and T. Boller. 1986. Plant chitinases are potent inhibitors of fungal growth. Nature 324:367.

Schroeder, C.A. 1968. Adventive embryogenesis in fruit pericarp tissue in vitro. Bot. Gaz. 129:374-376.

Sela-Buurlage, M.B., A.S. Ponstein, S.A. Bres Vloemans, L.S. Melchers, P.J.M. Van Den Elzen and B.J.C. Cornelissen. 1993. Only specific tobacco (*Nicotiana tabacum*) chitinases and β-1,3-glucanases exhibit antifungal activity. Plant Physiol. (Rockville) 101: 857-863.

Shapira, R., A. Ordentlich, H. Chet and A.B. Appenheim. 1989. Control of plant diseases by chitinase expressed from cloned DNA in *Escherichia coli*. Phytopathol. 79:1246-1249.

Shinshi, H., J.-M. Neuhaus, J. Ryals and F. Meins Jr. 1990. Structure of a tobacco endochitinase genes: Evidence that different chitinase genes can arise by transposition of sequences encoding a cyctein-rich domain. Plant Mol. Biol. 14: 357-368.

Singh, B.P. 1990. Cytogenetics and evolution in the Cucurbitaceae. p.11-28. In: D.M. Bates, R.W. Robinson and C. Jeffrey (eds.) Biology and Utilization of the Cucurbitaceae. Comstock Publ. Assoc., a division of Cornell Univ. Press, Ithaca.

Slightom, J.L., P.P. Chee and D. Gonzalves. 1990. Field testing of cucumber plants which express the CMV coat protein gene: Field plot design to test natural infection pressures, p.201-206. In: H.J.J. Nijkamp, L.H.W. Van der Plas and J. Van Aartrijk (eds.) Progress in Plant Cellular and Molecular Biology, Kluwer Acad, Publ., Dordrecht.

Songstad, D.D., C.L. Armstrong and W.L. Petersen. 1991. $AgNO_3$ increases type II callus production from immature embryos of maize inbred B73 and its derivatives. Plant Cell Rept. 9:699-702.

Stachel, S.E. and P.G. Zambryski. 1986. *Agrobacterium tumefaciens* and succeptible plant cells: A novel adaptation of extracellular recognition and DNA conjugation. Cell 47:155-157.

Staub, J.E. and R.S. Kupper. 1985. Use of *Cucumis sativus* var. *hardwickii* germplasm in backcrosses with *Cucumis sativus* var. *sativus*. HortSci. 20:436-438.

Stinzi, A., H. Heitz, V. Prasad, S. Weidemann-Merdinoglu, S. Kauffmann, P. Geoffroy, M. Legrand and B. Fritig. 1993. Plant 'pathogenesis-related'

proteins and their roles in defense against pathogens. Biochemie 75:687-706.

Swegle, M., K.J. Kramer and S. Muthukrishnan. 1992. Properties of barley seed chitinases and release of embryo-associated isoforms during early stages of imbibition. Plant Physiol. (Bethesda) 99:1009-1014.

Tang, F.A. and Z.K. Punja. 1989. Isolation and culture of protoplasts of *Cucumis sativus* and *Cucumis metuliferus* and methods for their fusion. Cucurbit Genet. Coop. Rept. 12: 29-34.

Trulson, A.J. and E.A. Shahin. 1986. In vitro plant regeneration in the genus *Cucumis*. Plant Sci. 47:35-43.

Trulson, A.J., R.B. Simpson and E.A. Shahin. 1986. Transformation of cucumber (*Cucumis sativus* L.) plant with *Agrobacterium rhizogenes*. Theor. Appl. Genet. 73:11-15.

Turk, S.C.H.J., L.S. Melchers, H. den Dulk-RasA.J.G. Regensgurg-Tuink, P.J.J. Hoykaas. 1991. Environmental conditions differentially effect *vir* gene induction in different *Agrobacterium* strains. Role of the VirA sensor protein. Plant Mol. Biol. : 1051-1059.

Van den Elzen, P.J.M., E. Jongedijk, L.S. Melchers and B.J.C. Cornelissen. 1993. Virus and fungal resistance: From laboratory to field. Philosophical Transactions of the Royal Society of London B Biological Sciences 342: 271-278.

Van der Mark, F., J.H.W. Bergervoet and J.B.M. Custers. 1989. Transformation of cucumber with *A. rhizogenes*. Cucurbit Genet. Coop. Rept. 12:35-36.

Vasseur, V., F. Arigoni, H. Andersen, G. Defago, G. Bompeix and J.-M. Seng. 1990. Isolation and charaterization of *Aphanocladium album* chitinase-overproducing mutants. J. Gen. Microbiol. 136:2561-2567.

Verburg, J.G. and Q.K. Huynh. 1991. Purification and characterization of an intifungal chitinase from *Arabidopsis thaliana*. Plant Physiol. 95:450-455.

Walden, R. 1988. Genetic Transformation in Plants. Open University Press, Milton Keynes. 138p.

Wang, K., L. Hurrere-Estrella, M. Van Montagu and P. Zambryiski. 1984. Right 25 bp terminus sequence of the nopaline T-DNA is essential for and determines direction of DNA transfer from *Agrobacterium* to the plant genome. Cell 38:455-462.

Wehner, T.C. and R.D. Locy. 1981. I n vitro adventitious shoot and root formation of cultivars and lines of *Cucumis sativus* L. HortSci. 16:759-760.

Wehner, T.C., R.M. Cade and R.D. Locy. 1990. Cell, tissue, and organ culture techniques for genetic improvement of cucurbits, p.367-381. In: D.M. Bates, R.W. Robinson and C. Jeffrey (eds.) Biology and Utilization of the Cucurbitaceae, Comstock Publ. Assoc., Ithaca

Williams, D., D.A.C. Pink and N.L. Bedington. 1990. Effect of silver nitrate on long-term culture and regeneration of callus from *Brassica oleracea* var. *gemmifera*. Plant Cell Tis. Org. Cult. 21:61-66.

Winans, S., R. Kerstetter, J. Ward and E.W.J. Nester. 1989. A protein required for transcriptional regulation of *Agrobacterium* virulence genes spans the cytoplasmic membrane. J. Bacteriol. 171: 1616-1622.

Yadava, K.S., A.K. Singh and H.C. Arya. 1984. Cytogenetic investigation in *Cucumis* L I. Meiotic analysis in twenty-four *Cucumis* species. Cytologia 49:1-9.

Zambryski, P.C. 1992. Chronicle fron the *Agrobacterium*-plant cell DNA transfer story. Annu. Rev. Plant Physiol. Plant Mol. Biol. 43:465-490.

Zambryski, P., J. Tempe and J. Schell. 1989. Transfer and function if T-DNA genes from *Agrobacterium* Ti and Ri plasmids in plants. Cell 56:193-201.

Zhang, Y-Y. and Z.K. Punja. 1994. Induction and characterization of chitinase isoforms in cucumber (*Cucumis sativus* L.): effect of elicitors, wounding and pathogen inoculation. Plant Sci. 99:141-150.

Zhu, Q., P.W. Doerner and C.J. Lamb. 1993. Stress induction and developmental regulation of a rice chitinase promoter in transgenic tobacco. Plant J. 3: 203-212.

Zhu, Q., E.A. Maher, S. Masoud, R.A. Dixon and C.J. Lamb. 1994. Ennhanced protection against fungal attack by constitutive co-expression of chitinase and glucanase genes in transgenic tobacco. Bio/Technol. 12: 807-812.

Ziv, M. and G. Gadasi. 1986. Enhanced embryogenesis and plant regeneration from cucumber (*Cucumis sativus* L.) callus by activated charcoal in solid/liquid double-layer cultures. Plant Sci. 47:115-122.

APPENDICES

APPENDIX I

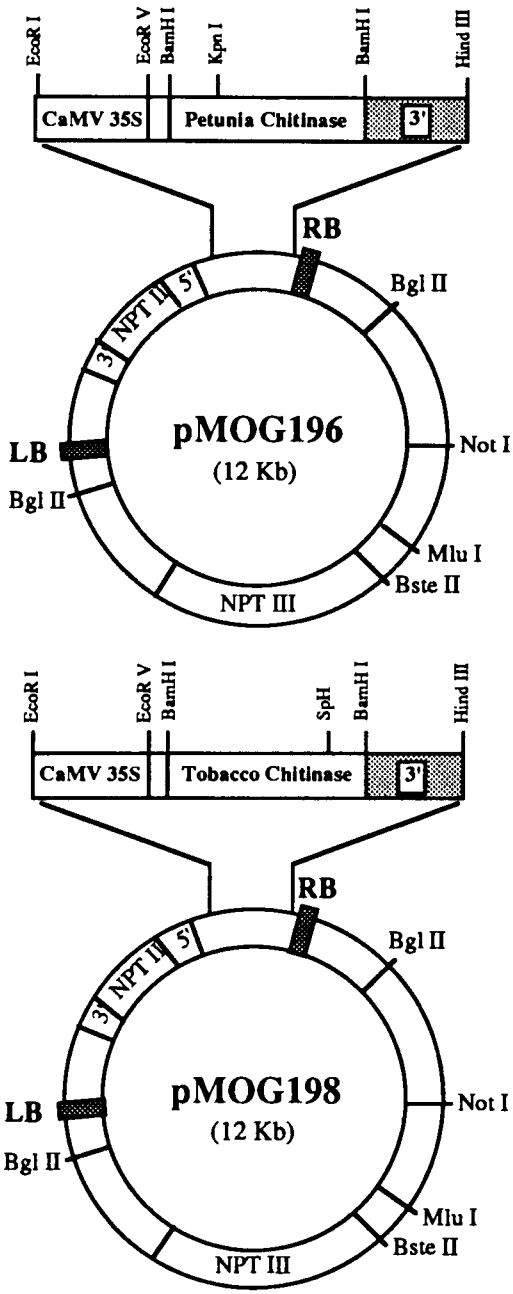


Figure A.1. Schematic diagram of the binary vectors pMOG196 (*top*) and pMOG198 (*bottom*) containing a petunia acidic or a tobacco basic chitinase gene, respectively (Linthorst *et al.*, 1990).

APPENDIX II

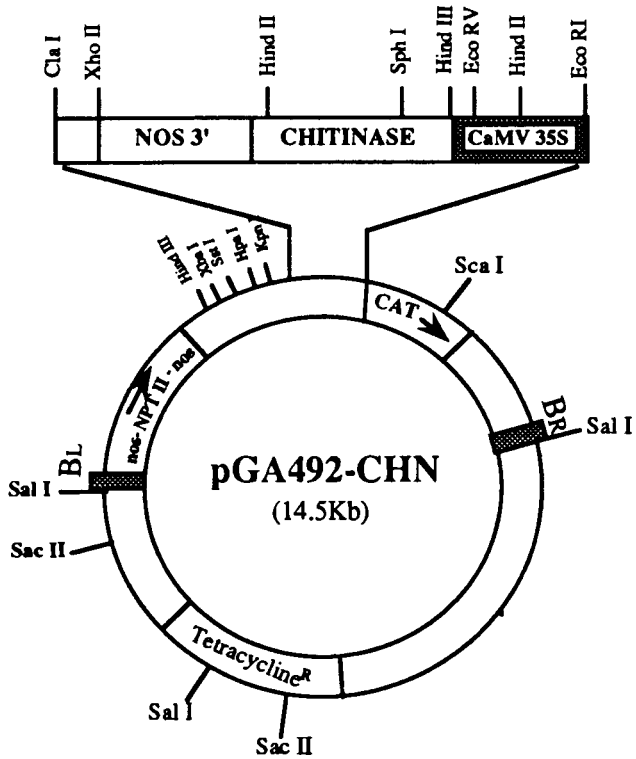


Figure A.2. Schematic diagram of the binary vector pGA492-CHN containing a bean basic chitinase gene under control of the CaMV *35S* promoter and NOS terminator (courtesy Hernandez, 1994)

APPENDIX III

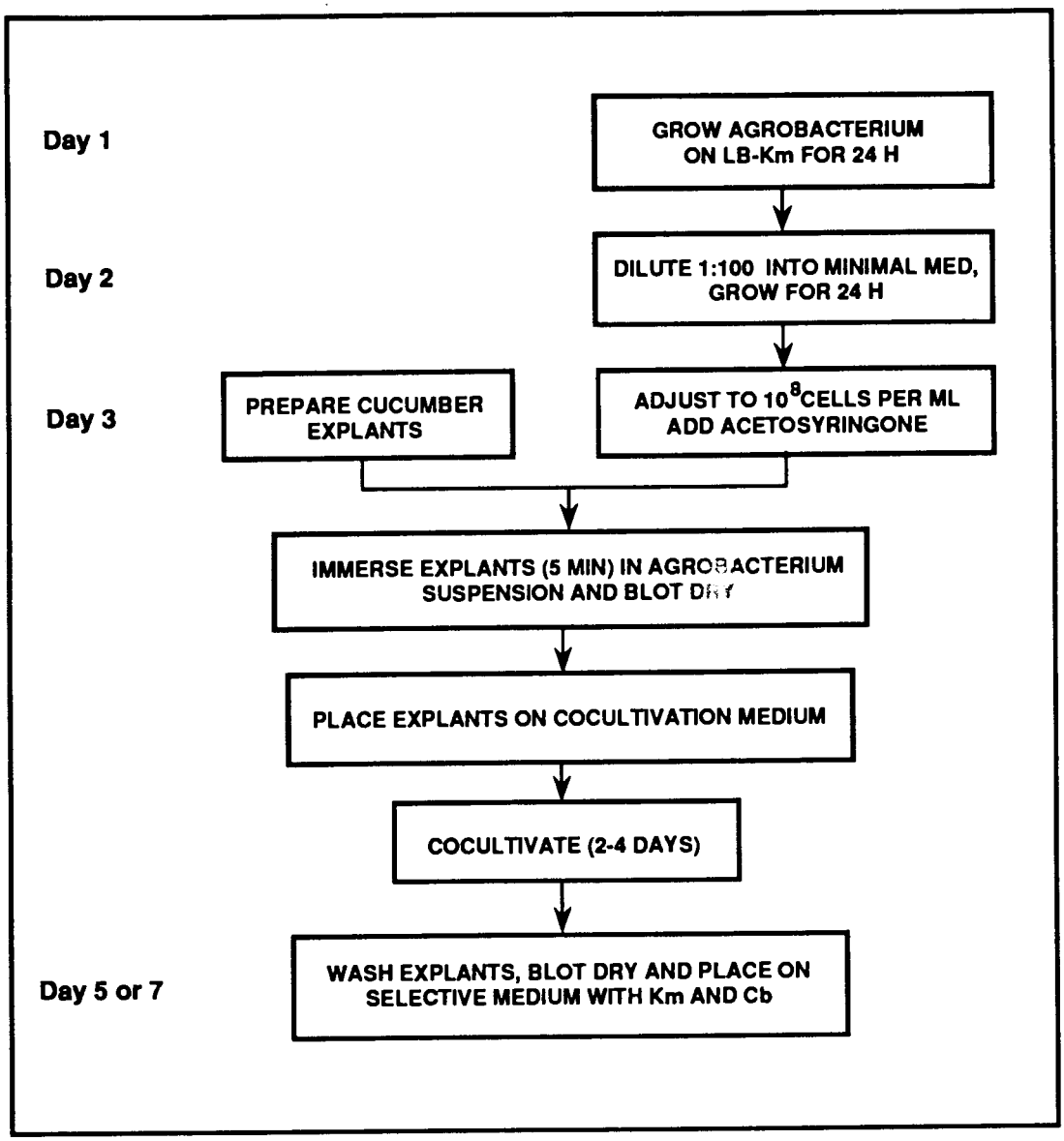


Figure A.3. Schematic diagram of the protocol for the infection and cocultivation of cucumber explants with *Agrobacterium tumecasiens.*

APPENDIX IV

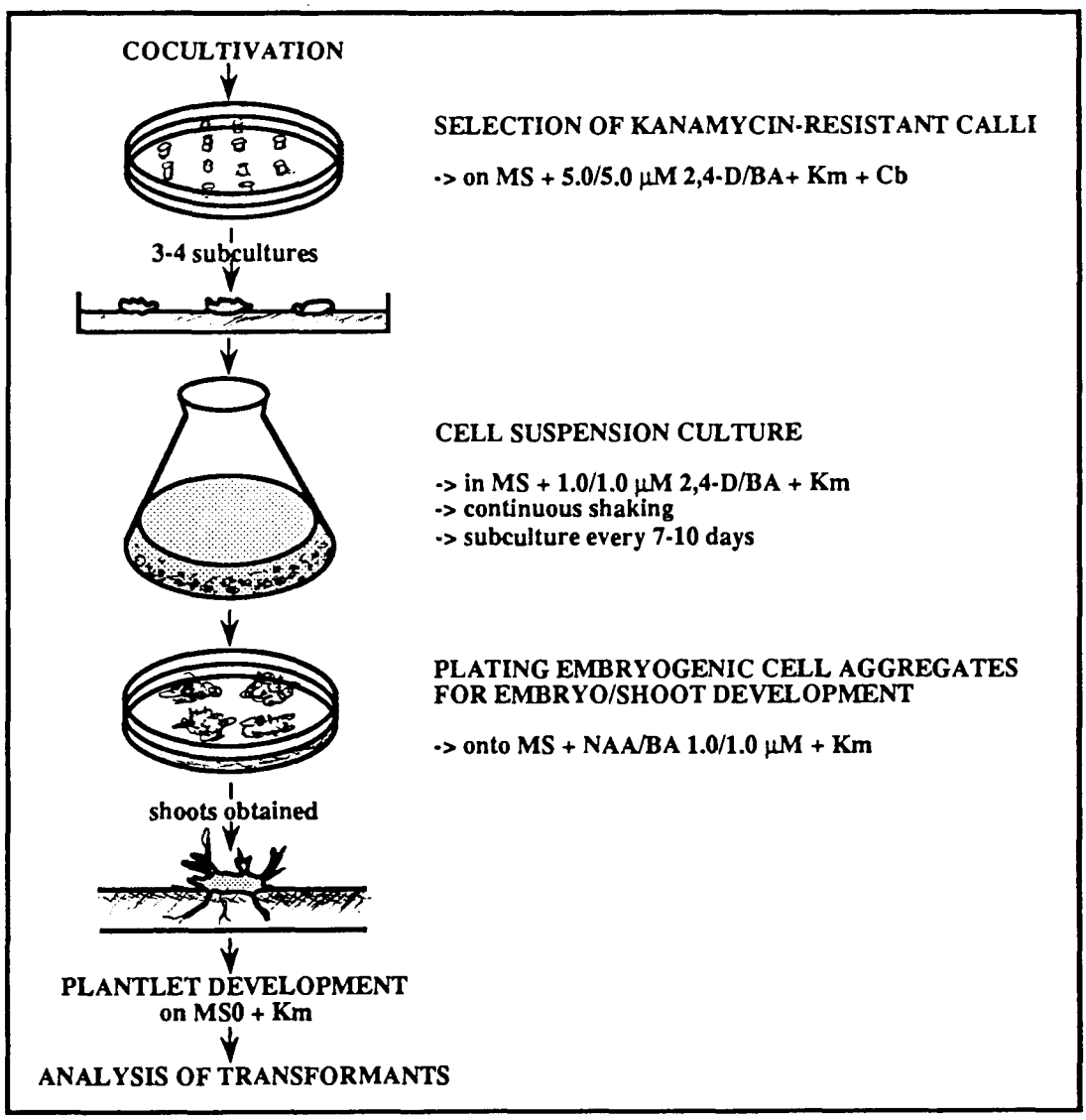


Figure A.4. Schematic diagram of the procedures for *Agrobacterium*-mediated transformation of cucumber cv. Endeavor, by involving selection and multiplication of kanamycin-resistant calli in cell suspension culture.