AN ABSTRACT OF THE THESIS OF

Yuexin Wang for the degree of Master of Science in Horticulture presented on May 20, 1998.

Title: Plant Regeneration from Cell Suspension Cultures of Iris (Iris germanica L. cv. 'Skating Party')

Abstract approved: ____________________________________

Tony H. H. Chen

This thesis reports a successful in vitro plant regeneration system for Iris germanica L. from cell suspension cultures. A series of experiments was conducted to systematically examine factors which critically affect induction of differentiated clumps from the suspension culture, as well as the induction of shoot and root regeneration from the clumps.

A stable suspension culture was established from callus induced on leaf bases and was maintained by subculturing every 3 weeks. Suspension cultures were maintained in Murashige and Skoog (MS) medium (pH 5.9) supplemented with 290 mg·l⁻¹ proline, 50 g·l⁻¹ sucrose, 5.0 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 μM kinetin (Kin). Suspension-cultured cells were transferred to a shoot induction medium, MS basal medium supplemented with 10 mg·l⁻¹ pantothenic acid, 4.5 mg·l⁻¹ nicotinic acid, 1.9 mg·l⁻¹ thiamin, 250 mg·l⁻¹ casein hydrolysate, 250 mg·l⁻¹ proline, 50 g·l⁻¹ sucrose, 2.0 g·l⁻¹ Phytagel™ (Sigma, St. Louis, MO), 0.5 μM NAA (α-Naphthaleneacetic acid), and 12.5 μM Kin. Cell clusters differentiated and eventually developed into shoots in 5 weeks. At the suspension culture
phase, the effects of plant growth regulators (2,4-D acid and Kin), length of culture period, and the size of multicellular aggregates on plant regeneration were examined. The highest regeneration efficiency was achieved when suspension cultures were grown in a liquid medium containing 5 μM 2,4-D and 0.5 μM Kin for 6 weeks, and the suspension cultures were then passed through a stainless steel sieve with pore size <280 μm in diameter. The screened cell fraction was inoculated onto an induction medium to induce plant regeneration.

Various combinations of Kin and NAA were tested for their effects on the induction of differentiated cellular structures and subsequent development. The most regenerable clumps (66.7%) and (63.0%) developing into both shoots and roots simultaneously were produced on induciton medium with 2.5 or 12.5 μM Kin with 0.5 μM NAA. Medium containing 1.25 μM N\textsuperscript{6}-benzyladenine (BA) were optimal for plant development from those regenerable clumps.

Rooted plantlets were easily transplanted and acclimatized to soil mixtures in pots for greenhouse growth. This represents an efficient in vitro plant regeneration system for iris, allowing regeneration of a large number of morphologically normal plants.
©Copyright by Yuexin Wang

May 20, 1998

All Rights Reserved
PLANT REGENERATION FROM CELL SUSPENSION CULTURES

OF IRIS (IRIS GERMANICA L. CV. 'SKATING PARTY')

by

Yuexin Wang

A Thesis

submitted to

Oregon State University

in partial fulfillment of

the requirements for the

degree of

Master of Science

Presented May 20, 1998
Commencement June, 1999
Master of Science thesis of Yuexin Wang presented on May 20, 1998

APPROVED:

Major Professor, representing Horticulture

Head of Department of Horticulture

Dean of Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Yuexin Wang, Author
ACKNOWLEDGMENTS

This thesis research could not have been accomplished without the support and help of many people. I would like to express my sincere appreciation to my major professor, Dr. Tony H. H. Chen, whose financial support, contributions of ideas, constructive criticism, and all other help made this research fruitful.

I would like to express my sincere gratitude to the members of my graduate committee: Dr. Christopher Mathews, Dr. Barbara Reed, and Dr. Ralph Berry for their valuable advice, encouragement, and their most precious time.

I am especially thankful to Dr. Bill Mansour for his friendship, encouragement, valuable suggestion, and kind correction of the first draft of my thesis; to Dr. Fred Rickson for his kindly allowing me to use the facilities of his laboratory and guiding me during my histological study, and also to Ms. Priscilla Licht for her close correction of this thesis.

My special appreciation goes to the many graduate students, faculty, and staff in the Department of Horticulture for their friendship and support, which have made a positive difference. I am especially grateful for the fellowship, friendships, and help from Dr. Candolphi Carinno, Fred Dixion, Sunghee Guak, Lailiang Cheng, and Rengong Meng. I am especially indebted to Lailiang Cheng and Sunghee Guak, who greatly helped me in computer application during data analysis and seminar preparation. I also appreciate the fellowship from all of our laboratory fellows who have closely helped me in many ways, especially Zoran Jecnic, who helped me in scanning microscopic analysis and preparing some of the figures.

I deeply appreciate so much the physical and spiritual support from my families, my father, late mother, my mother-in-law, and all of my brothers and sisters. Special thanks to my deeply loved wife, Yanping Chen, for all her immediate help in every possible way to make my study and work easier. And also to my daughter, Kai Wang, for her love, obedience, and consolatory progress and for the cheerful and joyous moments she shared with me at home.
TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW ..................................1

1.1 INTRODUCTION .........................................................................................1

1.2 PLANT REGENERATION FROM CALLUS CULTURE ..................................2

1.2.1 MAJOR FACTORS INFLUENCING CALLUS INITIATION AND PLANT
    REGENERATION .......................................................................................4

1.2.1.1 SOURCE OF EXPLANTS .................................................................4
1.2.1.2 MEDIUM COMPOSITION ..............................................................4
1.2.1.3 PLANT GROWTH REGULATORS ....................................................5

1.2.2 SHOOT AND/OR SOMATIC EMBRYO INDUCTION ................................6

1.3 PLANT REGENERATION FROM PROTOPLAST CULTURE .........................7

1.4 PLANT REGENERATION FROM CELL SUSPENSION CULTURE ..................9

1.4.1 MAJOR FACTORS INFLUENCING PLANT REGENERATION FROM SUSPENSION-
    CULTURED CELLS .................................................................................10

1.4.1.1 GENOTYPES AND EXPLANT SOURCES ........................................10
1.4.1.2 MEDIA ..........................................................................................11
1.4.1.3 PLANT GROWTH REGULATORS ....................................................12
1.4.1.4 LENGTH OF CULTURE PERIOD OF SUSPENSION CULTURE ..........13
1.4.1.5 SIZE OF MULTICELLULAR CELL AGGREGATES .................................13

1.4.2 INDUCTION OF SHOOT OR PLANTLET REGENERATION ...................13

1.5 PROBLEMS ...............................................................................................14

1.6 OBJECTIVES ............................................................................................16

1.7 LITERATURE CITED ..................................................................................16
<table>
<thead>
<tr>
<th>CHAPTER 2: FACTORS AFFECTING PLANT REGENERATION FROM SUSPENSION-CULTURED CELLS OF <em>IRIS GERMANICA</em> L.</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 ABSTRACT</td>
<td>22</td>
</tr>
<tr>
<td>2.2 INTRODUCTION</td>
<td>23</td>
</tr>
<tr>
<td>2.3 MATERIALS AND METHODS</td>
<td>24</td>
</tr>
<tr>
<td>2.3.1 ESTABLISHMENT AND MAINTENANCE OF SUSPENSION CULTURES</td>
<td>24</td>
</tr>
<tr>
<td>2.3.2 PLANT REGENERATION</td>
<td>25</td>
</tr>
<tr>
<td>2.3.3 SCANNING ELECTRON MICROSCOPY (SEM)</td>
<td>26</td>
</tr>
<tr>
<td>2.3.4 EFFECTS OF 2,4-D AND <em>K</em>in</td>
<td>26</td>
</tr>
<tr>
<td>2.3.5 EFFECT OF THE LENGTH OF THE NON-SUBCULTURE PERIOD</td>
<td>27</td>
</tr>
<tr>
<td>2.3.6 EFFECT OF THE SIZE OF CELL CLUSTERS</td>
<td>27</td>
</tr>
<tr>
<td>2.3.7 DATA COLLECTION AND ANALYSIS</td>
<td>27</td>
</tr>
<tr>
<td>2.4 RESULTS</td>
<td>28</td>
</tr>
<tr>
<td>2.4.1 ESTABLISHMENT AND MAINTENANCE OF SUSPENSION CULTURES</td>
<td>28</td>
</tr>
<tr>
<td>2.4.2 MORPHOGENESIS OF PLANT REGENERATION</td>
<td>28</td>
</tr>
<tr>
<td>2.4.3 EFFECTS OF 2,4-D AND <em>K</em>in COMBINATIONS</td>
<td>29</td>
</tr>
<tr>
<td>2.4.4 EFFECT OF THE LENGTH OF THE NON-SUBCULTURE PERIOD</td>
<td>34</td>
</tr>
<tr>
<td>2.4.5 EFFECT OF THE SIZE OF CELL CLUSTERS</td>
<td>34</td>
</tr>
<tr>
<td>2.5 DISCUSSION</td>
<td>40</td>
</tr>
<tr>
<td>2.6 LITERATURE CITED</td>
<td>45</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CHAPTER 3: OPTIMIZATION OF CONDITIONS FOR THE INDUCTION OF PLANT REGENERATION FROM SUSPENSION CULTURED-CELLS OF <em>IRIS GERMANICA</em> CV. 'SKATING PARTY'</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1 ABSTRACT</td>
<td>49</td>
</tr>
<tr>
<td>3.2 INTRODUCTION</td>
<td>49</td>
</tr>
<tr>
<td>3.3 MATERIALS AND METHODS</td>
<td>50</td>
</tr>
<tr>
<td>3.3.1 MEDIA</td>
<td>50</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>3.3.2 Preparation of Suspension Cultures for Regeneration Experiments</td>
<td>51</td>
</tr>
<tr>
<td>3.3.3 Effect of Kin and NAA Combinations in Induction Medium</td>
<td>51</td>
</tr>
<tr>
<td>3.3.4 Effect of BA Concentrations on Development of Differentiated Clumps</td>
<td>52</td>
</tr>
<tr>
<td>3.3.5 Relationship between size and age of differentiated clumps and their regeneration capability</td>
<td>53</td>
</tr>
<tr>
<td>3.3.6 Acclimatization and Substrates for Growing In Vitro Regenerated Iris Plants in the Greenhouse</td>
<td>53</td>
</tr>
<tr>
<td>3.4 Results</td>
<td>54</td>
</tr>
<tr>
<td>3.4.1 Effects of Kin and NAA Combinations in Induction Medium</td>
<td>54</td>
</tr>
<tr>
<td>3.4.2 Effects of Kin and NAA Combinations on the Subsequent Development of Regenerable Clumps</td>
<td>56</td>
</tr>
<tr>
<td>3.4.3 Effect of BA Concentrations on the Development of Differentiated Clumps</td>
<td>56</td>
</tr>
<tr>
<td>3.4.4 Relationship between size and age of differentiated clumps and their regeneration capability</td>
<td>60</td>
</tr>
<tr>
<td>3.4.5 Effects of Acclimatization Conditions and Substrates on Plant Establishment in the Greenhouse</td>
<td>65</td>
</tr>
<tr>
<td>3.5 Discussion</td>
<td>65</td>
</tr>
<tr>
<td>3.6 Literature Cited</td>
<td>68</td>
</tr>
<tr>
<td>Chapter 4: Conclusion</td>
<td>71</td>
</tr>
<tr>
<td>Bibliography</td>
<td>72</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
</tr>
<tr>
<td>2-1.</td>
<td>Morphogenesis process of plant regeneration of <em>I. germanica</em> cv. 'Skating Party'. An irregular, multicellular mass immediately after placement on solid induction medium (Fig. 2-1A). A few days to 2 weeks after being placed on induction medium, the recovered cell aggregates began to enlarge and became visually identifiable opaque callus (Fig. 2-1B). At 3 to 4 weeks after plating, a number of the callus underwent further growth and differentiation into the formation of differentiated clumps (Fig. 2-1C), from which numerous well-organized structures were formed (Fig 2-1D, 2-1E). These structures could initiate plantlets and shoots on the first developing medium (Fig. 2-1F) and the second BA-free developing medium (Fig 2-1G). The plantlets were easily acclimatized under greenhouse conditions (Fig 2-1H).</td>
</tr>
<tr>
<td>2-2.</td>
<td>Scanning electron micrographs of the early stages of plant regeneration from suspension cultures of <em>Iris germanica</em>. A. Development of embryo-like nodules, which appeared on visually identifiable opaque calli and organized as strata of cells a few days to 2 weeks after placement on induction medium. B. At 3 to 4 weeks after plating, globular, embryo-like structures developed from the embryo-like nodules. C. Elongated, globular, embryo-like structures and the developing shoot apices. D. A regenerated shoot. Bar sizes: A, B, and C 100 um, D 1 mm.</td>
</tr>
<tr>
<td>2-3.</td>
<td>Effects of plant growth regulator (2,4-D and Kin) combinations in a liquid medium. A. Number of differentiated clumps on an induction medium 5 weeks after inoculation. B. Number of regenerable clumps 5 weeks after being transferred to a solid developing medium.</td>
</tr>
<tr>
<td>2-4.</td>
<td>Effects of 2,4-D and Kin in a liquid medium on differentiation of shoots and roots. A. Number of both shoot- and root-borne regenerable clumps on a solid developing medium 5 weeks after transfer. B. Number of only shoot-borne regenerable clumps 5 weeks after being transferred to a solid developing medium.</td>
</tr>
<tr>
<td>2-5.</td>
<td>Effect of the length of the non-subculture period of suspension cultures on plant regeneration.</td>
</tr>
<tr>
<td>2-6.</td>
<td>Effect of the size of multicellular aggregates from suspension cultures on plant regeneration.</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
</tr>
<tr>
<td>3-1.</td>
<td>Effect of Kin and NAA combinations in IM on the quantity of differentiated clumps. After 6 weeks on IM, the recovered suspension cells differentiated into callus clumps on which white structures were born. Some of the structures are regenerable after being transferred to DM1 and DM2.</td>
</tr>
<tr>
<td>3-2.</td>
<td>Effect of BA in DM1 on development of differentiated clump induced from suspension cells on IM with 2.5 μM Kin and 0.5 μM NAA for 6 weeks. After 4 weeks, A) medium containing 1.25 μM BA showed well-developed shoots and roots; B) medium containing 2.5 μM BA showed poor root development from clumps.</td>
</tr>
<tr>
<td>3-3.</td>
<td>Size classification of differentiated clumps developed on IM 5 weeks after suspension cells were inoculated. Size classes are showed from bottom to top: large = &gt; 10 mm; Medium = ~5 to 10 mm; Small = ~ 2 to 5 mm; Very Small = &lt; 2 mm</td>
</tr>
<tr>
<td>3-4.</td>
<td>Difference in regeneration and development among size classes of the differentiated clumps 5 weeks after being transferred onto DM1. Size classes of differentiated clumps: A = large, B = medium, C = small, D = very small.</td>
</tr>
</tbody>
</table>
LIST OF TABLES

<table>
<thead>
<tr>
<th>Tables</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-1. Effects of Kin and NAA combinations in IM on the regeneration and development of differentiated clumps in terms of % regeneration and % development in the subsequent culture for 6 weeks on DM1 with 1.25 μM BA</td>
<td>57</td>
</tr>
<tr>
<td>3-2. Effects of BA concentration in DM1 on the regeneration and development of the differentiated clumps induced from IM containing 2.5 μM Kin and 0.5 μM NAA.</td>
<td>58</td>
</tr>
<tr>
<td>3-3. Size transition of differentiated clumps between classes on IM during week 6 through week 9</td>
<td>62</td>
</tr>
<tr>
<td>3-4. Relationship between age and size of differentiated clump from suspension cells incubated on IM with 2.5 μM Kin and 0.5 μM NAA for 6 weeks in terms of % regeneration and % clumps producing big shoots</td>
<td>64</td>
</tr>
<tr>
<td>3-5. Effect of substrates of greenhouse cultivation on plant recovery from transfer and acclimatization, and on growth in fresh weight after 6 month’s cultivation in greenhouse</td>
<td>66</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-D</td>
<td>2,4-dichlorophenoxyacetic acid</td>
</tr>
<tr>
<td>BA</td>
<td>N⁶-benzyladenine</td>
</tr>
<tr>
<td>DM1</td>
<td>The first developing medium containing 0.0 1.25 or 2.5 μM BA</td>
</tr>
<tr>
<td>DM2</td>
<td>The second developing medium containing no growth regulators</td>
</tr>
<tr>
<td>GA₃</td>
<td>Gibberellic acid</td>
</tr>
<tr>
<td>IAA</td>
<td>Indole-3-acetic acid</td>
</tr>
<tr>
<td>IBA</td>
<td>Indole-3-butyric acid</td>
</tr>
<tr>
<td>IM</td>
<td>Induction medium</td>
</tr>
<tr>
<td>Kin</td>
<td>Kinetin</td>
</tr>
<tr>
<td>MS</td>
<td>Murashige and Skoog medium</td>
</tr>
<tr>
<td>NAA</td>
<td>α-naphthaleneacetic acid</td>
</tr>
<tr>
<td>RH</td>
<td>Relative humidity</td>
</tr>
<tr>
<td>TIBA</td>
<td>2,3,5-triiodobenzoic acid</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Numerous varieties and cultivars of the Iris genus (Iridaceae family) are cultivated around the world. They are among the most fascinating of all plants both in the wild and in the garden. Even ancient people were attracted to these plants, especially in the Mediterranean regions. At the time of Theophrastes (372 to 287 BC), the beautiful plants with multi-colored flowers were given the name ‘Rainbow Goddess’. The pharaohs of ancient Egypt were supposed to have cultivated Iris susiana or a similar species in their gardens and iris were found on the island of Crete during the Minoan period, 2000 years before Christ. They also appeared very early in the cloister gardens of central Europe as ‘sword lilies’ or ‘flags’. The plant later called Iris germanica. was already known in Greece in Classical times (Kohlein, 1987).

In the early times, iris were primarily cultivated for medicinal purposes and for their fragrance. For example, they were used against snake bite or as a remedy for stomach and intestinal ailments. In ancient Greece, whole fields were planted with iris to harvest the expensive scented salve and other fragrant beauty products derived from the dried rhizomes (Kohlein, 1987). Today I. pallida is still greatly appreciated by perfumeries for the production of irones, violet-scented ketonic compounds, from rhizomes after long-term storage (Gozu et al., 1993, Jehan et al., 1994).
Iris is a winter-hardy, herbaceous perennial floral crop. The Genus *Iris* consists of approximately 300 species (Waddick and Zhao, 1992). The majority of the horticulturally important irises are bearded or pogon (hairy structures grow on the haft of petals) species, and their hybrids derived from species native to the Near East and Europe (Kohlein, 1987). They are now widely spread in the temperate regions of the Northern Hemisphere.

According to Mathew (1989), the *Iris* genus can be divided into 6 subgenera (*Iris, Limniris, Nepalensis, Xiphium, Scorpiris, and Hermodactyloides*). The subgenus *Iris* is further divided into 6 Sections (*Iris, Psammiris, Oncocyclus, Regelia, Hexapogon, and Pseudorelegia*). The Section *Iris* (the bearded or pogon iris) includes more than 100 species.

*Iris germanica*, a rhizomatic species, is one of the most horticulturally important tall bearded irises in the genus. Its origins will probably never be fully explained. It is likely a hybrid, rather than a pure species. Generally, the diploid *I. germanica* has to be regarded as an undefinable genetic conglomerate. Due to the traditional breeding efforts, the multiplicity of its forms and color variants has increased. As early as 350 years ago, Carolus Clusius had already described 28 different types of *I. germanica* (Kohlein, 1987). Today, hundreds of highly attractive cultivars of this species have been developed and cultivated commercially.

### 1.2 Plant Regeneration from Callus Culture

There are several important applications of *in vitro* plant regeneration in crop production and improvement (de Fossard., 1983; Hussey, 1983; George and Sherrington, 1984). (1) Through *in vitro* culture, the number of plants can be increased rapidly, thus
shortening the time needed for new cultivars to be marketed. (2) In vitro regeneration may accelerate the conventional breeding process via anther culture to obtain homozygosity, embryo rescue of a hybrid in an inter-specific cross, and multiplication of the desired individuals. (3) Efficient plant regeneration in vitro is the prerequisite to genetic transformation, a novel approach to improving the characteristics of existing cultivars. (4) In vitro culture also produces specific pathogen-free materials and offers a means of germplasm storage and exchange.

Plant regeneration can be achieved through organogenesis (e.g., adventitious shoot formation) or somatic embryogenesis, via callus culture, cell suspension culture, or protoplast culture. However, in vitro plant regeneration of monocotyledons has traditionally been viewed as more difficult than for dicotyledons (Krikorian and Katz, 1968); this is in large measure still justified, although there has been much progress recently, particularly with crops of agronomic significance (Kamo et al., 1990).

The feasibility of in vitro plant regeneration of iris was first demonstrated by Fujino et al. (1972). Since then, researchers have cultured callus of many species of Iris, using explant sources such as leaf bases, shoot apices, young inflorescence, flower parts, mature embryos, and rhizome apices (Meyer et al., 1975; Hussey, 1976; Reuther, 1977; Radojevic et al., 1987; Yabuya et al., 1991; Laublin et al., 1991; Radojevic and Subotic, 1992; Gozu et al., 1993; Jehan et al., 1994).
1.2.1 Major factors influencing callus initiation and plant regeneration

Major factors known to influence callus initiation or plant regeneration from callus culture of iris include the source of the explants, composition of the culture medium to initiate callus culture, and types and concentrations of plant growth regulators.

1.2.1.1 Source of explants

Source of explants exerts a significant influence on the establishment of callus culture. Among sources tested, the leaf bases, rhizome apices, and young inflorescence from young plant or flower pieces demonstrated a higher capability for producing regenerable callus (Gozu et al., 1993; Jehan et al., 1994). In addition, embryogenic callus was also induced on explants of shoots, mature embryos, and bulb scales (Reuther, 1977; van der Linde and Hol, 1988; van der Linde et al., 1988). However, only rhizogenesis occurred on the peduncles; no embryogenic callus was induced from anthers or roots (Jehan et al., 1994). Gozu et al. (1993) reported that only leaf base and rhizome explants sampled in October and November from field-grown plants showed success in embryogenic callus induction, whereas those sampled in July and August failed to produce embryogenic callus. This might imply a significant environmental effect on embryogenic callus induction (Gozu et al., 1993).

1.2.1.2 Medium composition

For most tissue culture work on iris species, MS basal medium (Murashige and Skoog, 1962) was most common, but the type and amount of sugar, gelling agents, vitamins, and growth regulators varied. For example, in embryo culture of *I. setosa,*
Radojevic and Subotic (1992) achieved their best results using MS medium with 5% (w/v) sucrose, 0.5 % (w/v) agar, 5 mg·l⁻¹ nicotinic acid, 10 mg·l⁻¹ pantothenic acid, 2 mg·l⁻¹ vitamin B-1, 100 mg·l⁻¹ myo-inositol, and 250 mg·l⁻¹ casein hydrolysate.

Jehan et al. (1994) reported optimal somatic embryogenic callus induction and plant regeneration from leaf bases and flower pieces of *I. germanica*, cultured on MS basal medium with 2.9 g·l⁻¹ proline and 50 g·l⁻¹ sucrose. The addition of proline significantly enhanced the production of embryogenic callus.

Shimizu et al. (1996) observed somatic embryogenesis from leaf base explants cultured in MS basal medium supplemented with 200 mg·l⁻¹ casein hydrolysate, 250 mg·l⁻¹ proline, 30 mg·l⁻¹ sucrose, and 2.5 mg·l⁻¹ gellan gum.

1.2.1.3 PLANT GROWTH REGULATORS

Plant growth regulators, used singularly or in combination, are essential for successful plant regeneration *in vitro*. There are 5 major types of ‘classical’ plant growth regulators: auxin, cytokinin, gibberellic acid, abscisic acid, and ethylene. These hormonal substances play critical roles in the growth and differentiation of plant cells.

Among auxins, 2,4-D, alone, or in combination with other growth regulators, is the most effective somatic embryogenic callus or somatic embryo inducer for all explants of tested *Iris* species (Radojevic et al., 1987; Laublin et al., 1991; Radojevic and Subotic, 1992; Gozu et al., 1993; Jehan et al., 1994; Shimizu et al., 1996). The optimal 2,4-D concentrations used by these authors varied from 1 to 5 mg·l⁻¹ although callus induced on medium containing 1 to 2 mg·l⁻¹ 2,4-D alone gave rise to good plant regeneration (Radojevic et al., 1987; Gozu et al., 1993; Jehan et al., 1994; Shimizu et al., 1996).
However, others found that combining 2,4-D with other auxins gave better results. Laublin et al. (1991) compared two induction media that contained either 1 or 5 mg·l$^{-1}$ 2,4-D plus 1 mg·l$^{-1}$ NAA, respectively, and found that the 2,4-D concentration of 1 mg·l$^{-1}$ plus 1 mg·l$^{-1}$ NAA was consistently suitable for callus induction and its subsequent rapid growth. Meyer et al. (1975) showed that just 2.5 mg·l$^{-1}$ NAA was enough for callus growth.

In all studies on iris tissue cultures, Kin was the preferred cytokinin (Meyer et al. 1975; Radojevic et al., 1987; Laublin et al., 1991; Radojevic and Subotic, 1992; Gozu et al., 1993; Jehan et al., 1994; Shimizu et al., 1996). Kin concentrations ranging from 0.1 to 1.0 mg·l$^{-1}$ were most suitable for somatic embryogenic callus induction and/or plant regeneration (Laublin et al., 1991; Radojevic and Subotic, 1992; Gozu et al., 1993; Shimizu et al., 1996).

1.2.2 SHOOT AND/OR SOMATIC EMBRYO INDUCTION

Plant regeneration occurred during callus induction (Meyer et al., 1975; Gozu et al., 1993) or when the callus was cultured under an inductive condition (Radojevic et al., 1987; Laublin et al., 1991; Radojevic and Subotic, 1992; Jehan et al. 1994). Different types and concentrations of plant growth regulators were applied in the media to induce somatic embryos and/or shoots from the callus and, subsequently, to induce roots. Compared to callus initiation, a much more diluted concentration of both auxin (2,4-D, IBA, or IAA) and Kin was subsequently needed in the induction and rooting events. Radojevic et al. (1987) applied a liquid MS medium containing combinations of 2.0 mg·l$^{-1}$ GA$_3$, 2.0 mg·l$^{-1}$ IAA, and 1.0 mg·l$^{-1}$ IBA to allow somatic embryos formed on
regenerable callus to germinate; later on, however, they used a medium containing only 0.1 mg·l⁻¹ each of 2,4-D and Kin (Radojevic and Subotic, 1992). Jehan et al. (1994) obtained the best plant regeneration after transferring the undifferentiated callus to KN medium (Knudson, 1946) with or without growth regulators, in which Phytagei™ (Sigma, St. Louis, MO) replaced the agar. This significantly reduced hyperhydricity, a physiological abnormality of in vitro-cultured explants or regenerants, which appear thick, shiny, and watery and are difficult to develop further. Shimizu et al. (1996) obtained the best plant regeneration on hormone-free MS medium, whereas Gozu et al. (1993) achieved the best regeneration on MS medium containing 1.0 mg·l⁻¹ Kin and 0.1 mg·l⁻¹ IBA.

In addition, a genotype-dependent response of plant regeneration was also observed for Iris. Laublin et al. (1991) tested 7 genotypes of I. versicolor and I. pseudacorus on MS basal medium supplemented with 4 different plant growth regulator combinations: 9 µM BA alone; 2 µM TIBA plus 5 µM Kin; 22 µM BA alone; or 4 µM TIBA plus 9 µM BA. The response of each genotype was greatly different on the 4 media in terms of callus growth and plant regeneration. A similar phenomenon was also reported for Gladiolus (Kamo et al., 1990).

1.3 PLANT REGENERATION FROM PROTOPLAST CULTURE

Protoplast culture has been considered an alternative approach for genetic transformation, provided that protoplasts are capable of cell division, colony formation, and plant regeneration from protoplast-derived cells. In almost all monocots, protoplasts isolated from regenerable suspension cells have the capacity to grow back to plants; these
include *Pennisetum* spp. (Vasil and Vasil, 1980; Vasil et al., 1983), *Oryza sativa* (Toriyama et al., 1988; Shimamoto et al., 1989), *Dactylis glomerata* (Horn et al., 1988), *Panicum* spp. (Lu et al., 1981; Heyser, 1984), *Saccharum officinarum* (Srinivasan and Vasil, 1986), *Zea mays* (Rhodes et al. 1988; Prioli and Sondahl, 1989; Shillito et al., 1989), *Festuca arundinacea* and *Lolium perenne* (Dalton, 1988), *Triticum aestivum* (Harris et al., 1988) and *Hemerocalluss* (Ling and Sauve, 1995). Plant species in which suspension cells capable of plant regeneration have been reported but from which protoplasts have not yet regenerated plants include *Gladiolus* (Kamo et al., 1990), *Musa* spp. (Novak et al., 1989).

Shimizu et al. (1996) reported the success of plant regeneration in protoplast cultures of *I. germanica* cv. 'Gl'. Segments of leaf bases containing apices were cultured on MS medium supplemented with 2 mg·l\(^{-1}\) 2,4-D, 0.1 mg·l\(^{-1}\)Kin, 200 mg·l\(^{-1}\) casein hydrolysate, 250 mg·l\(^{-1}\) proline, 30 g·l\(^{-1}\) sucrose, and 2.5 g·l\(^{-1}\) gellan gum, to induce callus formation. To induce suspension cultures, selected friable embryogenic callus was transferred to a liquid N6 medium (Chu et al., 1975) with 1 mg·l\(^{-1}\) each of 2,4-D and Kin. This medium was supplemented with the same amounts of the agents as in the callus induction medium, but without gellan gum. The weekly-subcultured suspension cultures were used as the source material for protoplast isolation (Gleddie et al., 1986). A series of glucose concentrations, different types of sugars, and combinations of 2,4-D and Kin were examined for their effects on protoplast division and colony formation during protoplast culture. The N6 liquid medium was supplemented with 200 mg·l\(^{-1}\) casein hydrolysate, 250 mg·l\(^{-1}\) proline, and 20 g·l\(^{-1}\) agarose. The medium containing 0.2 M
glucose or sucrose, 0.1 or 1 mg·l\(^{-1}\) 2,4-D, and 1 mg·l\(^{-1}\) Kin, produced the highest frequency of division (21.1\%) and was also the most effective for colony formation. When the colonies were transferred onto hormone-free MS medium, somatic embryos were produced. However, the efficiency of plant regeneration from protoplast culture was not very high (Shimizu et al., 1996).

1.4 PLANT REGENERATION FROM CELL SUSPENSION CULTURE

Plant regeneration through suspension culture has advantages over callus culture. It provides a convenient means for studying the behavior of isolated single cells or small cell aggregates in the process of their growth, differentiation, and regeneration. Millions of cells or cell aggregates can easily be maintained in a single culture vessel and are easily multiplied by dividing the cultures and routinely subculturing them. In addition, suspension-cultured cells or cell aggregates can be induced to produce a large number of plantlets in a short time. This is especially true for monocots, in which in vitro plant regeneration is generally more difficult to obtain (Krikorian and Katz, 1968; Wang and Nguyen, 1990; Kamo et al., 1995).

Many researchers have emphasized the importance of establishing cell suspensions because it has been found with almost all monocots that regenerable suspension cells generally have the highest capacity for plant regeneration (Novak et al., 1989; Tsukahara et al., 1996). Protoplasts derived from suspension-cultured cells were once considered an important tool for genetic transformation of monocots (Vasil and Vasil, 1980; Lu et al., 1981; Vasil et al., 1983; Heyser, 1984; Srinivasan and Vasil, 1986; Dalton, 1988; Harris et al., 1988; Horn et al., 1988; Rhodes
et al., 1988; 1988; Toriyama et al., 1988; Prioli and Sondahl, 1989; Shillito et al., 1989; Shimamoto et al., 1989; Wang and Nguyen, 1990). To date, however, successful plant regeneration via suspension-cultured cells has been reported only in a few monocot ornamentals (Krikorian and Kann, 1981; Kamo et al., 1990).

The successful establishment of iris suspension culture was achieved in our laboratory in 1993 (T. Chen, unpublished) and in Japan (Shimizu et al., 1996). Though plant regeneration through cell suspension culture of iris was possible, the efficiency was relatively low (Shimizu et al., 1997). Factors which might critically affect plant regeneration from suspension cultures have remained unclear. Plant regeneration from suspension-cultured cells generally involves 4 steps: (1) initiation of friable callus; (2) establishment of the suspension culture; (3) induction of somatic embryogenesis or organogenesis; and (4) shoot and root development.

1.4.1 MAJOR FACTORS INFLUENCING PLANT REGENERATION FROM SUSPENSION-CULTURED CELLS

1.4.1.1 GENOTYPES AND EXPLANT SOURCES

In many plant species, there is a distinguishable difference among genotypes and explant sources of a particular genotype in the ability to induce friable callus. For instance, in *Gladiolus*, Kamo et al. (1990) found that cultured inflorescence stalks of 'Blue Isle' and 'Hunting Song' produced only compact callus, which was not dissociable in a liquid medium. In contrast, a friable type of callus was initiated from cormels or tissue culture plantlets of cultivars ‘Peter Pears’, ‘Rosa Supreme’, and ‘Jenny Lee’.
Among the latter 3, ‘Peter Pears’ was especially easy for obtaining friable callus and was the only one that subsequently regenerated plants on a hormone-free solid medium. In *Iris*, Shimizu et al. (1996) also confirmed this phenomenon in establishing a cell suspension culture.

Friable callus is usually considered a prerequisite for establishing a cell suspension culture. However, there are exceptions. For example, Lu and Vasil (1981) and Vasil and Vasil (1982) were able to establish cell suspension cultures successfully from compact callus of *Panicum maximum* and *Pennisetum americanum*, respectively.

1.4.1.2 Media

Shimizu et al. (1996) successfully established a suspension culture of *Iris germanica*, initiating friable callus on MS agar medium and using N6 liquid medium (Chu et al., 1975). Others have used MS medium for both steps in other monocot crops (Halperin, 1966; Kamo et al., 1990; Wang and Nguyen, 1990). Once established, the suspension cultures were usually maintained in the same medium via regular subculturing (Halperin, 1966; Smith and Street, 1974; Kamo et al., 1990; Shimizu et al., 1996). Plant regeneration is generally accomplished on an agar medium containing the same inorganic materials as those for suspension culture (Halperin, 1966; Kamo et al., 1990; Shimizu et al., 1996), or with some modifications.

Sucrose has been the carbon source most often used (Halperin, 1966; Kamo et al., 1990; Shimizu et al., 1996); amounts have ranged from 0.2 to 0.3%. In callus culture of *Iris germanica*, Jehan et al. (1994) showed that callus derived from a medium containing 5% sucrose gave rise to the best regeneration. The pH of the medium was generally
adjusted from 5.5 to 5.9 (Smith and Street, 1974; Kamo et al., 1990; Shimizu et al., 1996) before autoclaving. In addition, various organic compounds were added to the culture media to promote plant regeneration. These included casein hydrolysate, proline (Shimizu et al., 1996), glycine, thiamine, pyridoxine, nicotinic acid (Kamo et al., 1990), and myo-inositol (Smith and Street, 1974; Kamo et al., 1990).

1.4.1.3 Plant Growth Regulators

Plant growth regulators exert a most critical influence in each of the steps of in vitro plant regeneration. Shimizu et al. (1996) used 2 mg·l⁻¹ 2,4-D and 0.1 mg·l⁻¹ Kin to initiate friable callus from leaf bases of L. germanica cv. 'G1'. However, for establishing suspension cultures, 2,4-D was reduced to 1 mg·l⁻¹ and Kin was increased to 1-mg·l⁻¹. The plant growth regulators were then completely removed from the induction medium to facilitate plant regeneration (Shimizu et al., 1996).

Decreasing the auxin concentration while increasing Kin in a suspension culture medium (compared with a callus initiation medium) generally enhanced cell growth in suspension cultures (Kamo et al., 1990; Tsukahara et al., 1996) and the initiation of proembryogenic cells (Halperin, 1966). To maintain the regeneration potential of the suspension culture, the concentration of 2,4-D is critical. Vasil (1988) suggested that levels of 1 to 2.5 mg·l⁻¹ 2,4-D were a major factor in preserving the embryogenic nature of wheat cultures. Further differentiation of the proembryogenic callus into mature regenerated structures took place only after the 2,4-D was removed from the medium (Fujimura and Komamine, 1980).
1.4.1.4 Length of non-subculture period of suspension culture

The interval for subculture of suspension cultures depends on the plant genotypes and usually ranges from 1 to 4 weeks (Kamo et al., 1990; Wang and Nguyen, 1990; Shimizu et al., 1996). Almost all studies on plant regeneration from suspension cultures have focused on the type and concentration of growth regulators, medium compositions and concentration, and culture conditions. There are no reports on how the age of the suspension cultures (length of non-subculture period after last subculture) affects its regeneration capability.

1.4.1.5 SIZE OF MULTICELLULAR CELL AGGREGATES

Halperin et al. (1966) examined the plant regeneration capability of different-sized fractions of wild carrot suspension cultures. They found that the 45-75μ fraction (pore size of the sieve used), consisting of free cells and proembryogenic masses of various size and shape, yielded the highest number of embryos. The <45μ fraction, containing mostly single cells and a small percentage of multicellular aggregate units of about 2-5 cells, developed a much lower number of embryos. Though the exact mechanism is unknown, the size of multicellular aggregates in the suspension culture is an important factor affecting plant regeneration efficiency.

1.4.2 INDUCTION OF SHOOT OR PLANTLET REGENERATION

Plant regeneration can be induced either on a liquid induction medium or on a solid medium. For instance, rice was successfully regenerated directly from a cell suspension culture (Ozawa and Komamine, 1989; Biswas and Zapata, 1992; Kobayashi
et al., 1992). However, Ozawa and Komamine (1989) also reported that regeneration frequency in the liquid medium was far lower (48%) than that of the semi-solid medium (93%). For 2 monocot ornamentals, *Hemerocallis* and *Gladiolus*, both shoots and somatic embryos were induced by transferring suspension culture cells to an agar induction medium (Krikorian and Kann, 1981; Kamo et al., 1990; Smith and Krikorian, 1991; Ling and Sauve, 1995).

1.5 PROBLEMS

Initiation of regenerable callus from explants excised from the parent plant is the first step in plant regeneration. However, the availability and suitability of explants varies by the season. For instance, inflorescence and flower parts are strictly limited by the blooming season; leaf bases in different seasons respond differently on agar media (Gozu et al., 1993). Contamination in explants may also cause tremendous extra work and waste. Unfortunately, the rate of producing regenerable callus while limiting contamination is not completely predictable nor easily controlled.

Production and maintenance of vigorous callus from monocots to ensure continuous plant regeneration have traditionally been difficult (Krikorian and Katz, 1968); this is certainly true for *Iris*. Provided that the regenerable callus, which have sporadically occur on uncontaminated explants, can be obtained, multiplying callus on an agar medium and maintaining the regeneration potential is a problem. In addition, the growth rate of iris callus on agar media is too slow to meet the need for mass plant regeneration or for genetic transformation.
In iris regeneration, most researchers use the term “somatic embryogenesis” without offering a careful anatomical analysis (Radojevic et al., 1987; Laublin et al., 1991; Jehan et al., 1994). In *Iris germanica*, no anatomical analysis work has been done so far to confirm the path of *in vitro* plant regeneration.

Though *in vitro* regeneration of iris has been achieved via callus culture and protoplast culture from various explants, the regeneration efficiency is generally very poor because of low callus induction rates and the low rate of regenerable callus. For instance, Radojevic et al. (1987) cultured 50 mature embryos; only 3 produced somatic embryogenic callus (6%), from which 7 plantlets were obtained. Shimizu et al. (1996) obtained only 100 plantlets from their entire experiment on protoplast culture. Gozu et al. (1993) expected only 500 plantlets per 6 months from the rhizome explant of 1 parent plant. Jiang and Xie (1995) estimated production of, at best, 1500 to 2000 plantlets per year under laboratory conditions. So far, Jehan et al. (1994) has been the most successful, producing 1800 plantlets from 150 mg of specifically isolated embryogenic callus in 8 months after initial explant inoculation. Nevertheless, this amount of embryogenic callus had to be derived from a large number of explants because the induction rate was only 1.5 to 3.0%. The decline in regeneration potential caused by repeatedly subculturing callus on agar medium may also be a problem. Therefore, to regenerate many plantlets, the explants from the parent plant must be taken repeatedly from the field. Even then, the callus induction may still fail because of seasonal effects on the physiological status of the plants (Gozu et al., 1993).

In addition to conventional breeding, genetic transformation offers an alternative approach to improving traits, e.g., resistance to herbicides, diseases, and insects, or
obtaining new floral colors (van Marrewijk, 1994). Recently, transgenic plants from several agronomically important monocots (corn, wheat, rice, oat, and sugarcane), have been obtained via particle bombardment of either regenerable suspension cells or regenerable callus (Fromm et al., 1990; Gordon-Kamm et al., 1990; Christou et al., 1991; Bower and Birch, 1992; Somers et al., 1992; Weeks et al., 1993). However, there are no reports on genetic transformation of Iris, probably because of the lack of an efficient plant regeneration system.

1.6 OBJECTIVES

The objectives of this thesis were:

1. To examine factors which affect plant regeneration from suspension-cultured Iris cells.

2. To optimize the induction conditions for in vitro plant regeneration of Iris.

1.7 LITERATURE CITED


CHAPTER 2

FACTORS AFFECTING PLANT REGENERATION FROM SUSPENSION-CULTURED CELLS OF *IRIS GERMANICA* L. CV. 'SKATING PARTY'

2.1 ABSTRACT

A protocol for efficient plant regeneration from suspension cultures of *Iris germanica* was developed. Suspension cultures were maintained in Murashige and Skoog (MS) medium (pH 5.9) supplemented with 290 mg·l\(^{-1}\) proline, 50 g·l\(^{-1}\) sucrose, 5.0 µM 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 µM kinetin (Kin). Suspension-cultured cells were transferred to a shoot induction medium, MS basal medium supplemented with 10 mg·l\(^{-1}\) pantothenic acid, 4.5 mg·l\(^{-1}\) nicotinic acid, 1.9 mg·l\(^{-1}\) thiamin, 250 mg·l\(^{-1}\) casein hydrolysate, 250 mg·l\(^{-1}\) proline, 50 g·l\(^{-1}\) sucrose, 2.0 g·l\(^{-1}\) Phytagel™ (Sigma, St. Louis, MO), 0.5 µM NAA, and 12.5 µM Kin. Experiments were conducted to optimize the parameters of suspension cultures that affected subsequent plant regeneration in the shoot induction medium. These parameters included combinations of 2,4-D and Kin concentrations, the interval of subculturing, and the size of the cell clusters. The highest regeneration rate was from suspension cultures grown in liquid MS medium containing 5 µM 2,4-D and 0.5 µM Kin for 6 weeks. The suspension cultures were then passed through a stainless screen (mesh 30) to select cell clusters with diameters < 520 µm. The screened cell fraction was inoculated onto a solid induction MS developing medium with 2,4-D and Kin (cell clusters differentiated and eventually developed into shoots in 5 weeks.). This proved to be a rapid and efficient *in vitro* plant regeneration system, which allowed us to produce a large number of iris plants with normal vegetative growth and morphology.


2.2 INTRODUCTION

_Iris germanica_ is one of the most horticulturally important tall bearded irises in the genus. Hundreds of valuable cultivars from this species have been developed and cultivated commercially as perennial ornamental plants. A strong consumer demand means increased challenges in developing new cultivars with novel flower characteristics. Thus the search for an alternative breeding method is imperative. The introduction of desirable traits by genetic transformation offers an attractive means for iris cultivar improvement.

The ability to regenerate plants from somatic tissues is generally considered a prerequisite for genetic transformation. In the last several decades many efforts have been made to induce plant regeneration via _in vitro_ callus culture of various explants from a number of _Iris_ species (Fujino et al., 1972; Meyer et al., 1975; Hussey, 1976; Radojevic et al., 1987; van der Linde and Hol, 1988; van der Linde et al., 1988; Radojevic and Landre, 1990; Laublin et al., 1991; Yabuya et al., 1991; Radojevic and Subotic, 1992; Gozu et al., 1993). In _I. germanica_, Reuther (1977) induced embryogenic callus from zygotic embryos; Jehan et al. (1994) regenerated plants via somatic embryogenesis from leaves, apices, and immature flowers. Shimizu et al. (1996) had success in protoplast culture and plant regeneration via somatic embryogenesis. Shimizu et al. (1997) induced embryogenic callus from 3 cultivars of _I. Germanica_, but obtained regenerable suspension cultures from only 1 of the 3. Unfortunately, efforts are still hindered by low efficiency of plant regeneration in _I. germanica_ and other _Iris_ species, such that a suitable regeneration system is not yet available for genetic transformation.
With the long-term goal of applying genetic engineering to iris cultivar development, we wished to establish a stable suspension culture capable of plant regeneration. Several parameters were systematically tested for their specific effects on regeneration from suspension-cultured iris cells. Based on these results, we then proceeded to develop an efficient system for plant regeneration from suspension cultures of *Iris germanica* cv. 'Skating Party'. This system can then be used to develop a reliable genetic transformation method for iris.

2.3 MATERIALS AND METHODS

2.3.1 ESTABLISHMENT AND MAINTENANCE OF SUSPENSION CULTURES

Greenhouse-grown plants of *Iris germanica* cv. 'Skating Party' were used as source material. Young shoots, approximately 40 to 50 mm tall, were excised from the stock plants, and 2 to 3 of the outer most leaves were removed from each shoot. The basal portions were excised and washed thoroughly with tap water, then immersed in 75% ethanol for 1 min, followed by immersion in 1.05% sodium hypochlorite [20% (v/v) Clorox] containing Tween 20 (2 to 3 drops/100 ml). They were gently shaken on a rotary shaker (100 rpm) for 25 min, then rinsed 3 times with sterile water. Afterward, individual leaves were carefully separated from the shoot and each leaf was sliced into approximately 5-mm-thick pieces. These explants were placed on Murashige and Skoog (1962) medium with 290 mg·l⁻¹ proline, 50 g·l⁻¹ sucrose, 2.0 g·l⁻¹ Phytagel™ (Sigma, St. Louis, MO), 5.0 µM 2,4-D, and 0.5 µM Kin (pH of the medium adjusted to 5.9 before
autoclaving). Callus was cultured at 25°C, in the dark, and subcultured every 3 weeks on the same type of medium.

To establish suspension cultures, callus was transferred to a liquid medium, incubated in the dark on a rotary shaker at 100 rpm, and subcultured monthly. The components of the liquid medium were the same as those of the callus induction medium, but without Phytagel™.

2.3.2 Plant regeneration

Six-week-old suspension cultures were filtered through a stainless screen (Sigma, St. Louis, MO, U.S.A), mesh 30, to remove large cell aggregates. The pass-through fraction was collected in 50-ml sterile tubes and centrifuged at 2500 rpm for 5 min in a clinical centrifuge (IEC, Model HN-SII; International Equipment Co., Needham Heights, Mass.). The pelleted cells were weighed and resuspended to density 0.2 g·mL⁻¹ in a liquid medium with same composition as the subsequent solid induction medium except gelling agent. A 0.5-ml aliquot was inoculated into a 15- × 60-mm sterile plastic plate containing 20 ml of the induction medium (IM). IM was MS basal medium (1962) supplemented with 10 mg·L⁻¹ pantothenic acid, 4.5 mg·L⁻¹ nicotinic acid, 1.9 mg·L⁻¹ thiamin, 250 mg·L⁻¹ casein hydrolysate, 250 mg·L⁻¹ proline, 50 g·L⁻¹ sucrose, 2.0 g·L⁻¹ Phytagel™, 0.5 μM NAA, and 12.5 μM Kin. The plates were incubated in the dark at 25°C for 5 weeks. In each experiments, 5 plates were used for each replicate, and each entire experiment was repeated twice.

The clumps of induced structures were then transferred to Magenta GA-7 vessels (Sigma) with 50 ml of a developing medium (DM1) containing the same components as
the induction medium, except that NAA and Kin were replaced with 1.25 μM BA. Clumps were cultured at 23°C under light (ca. 50 μmol m⁻² s⁻¹ for 16h/24h) for another 6 weeks, to allow shoot and root development. Clumps with or without shoots were then transferred to plant growth regulator-free MS medium (DM2) for 5 more weeks for further root development under the same cultural conditions. Afterward, plantlets were moved to the greenhouse for acclimatization, and were eventually established in soil mixture in 6" plastic pots in greenhouse (see next chapter).

2.3.3 Scanning Electron Microscopy (SEM)

Samples at several different stages of differentiation were excised from tissues grown on IM. They were fixed in 2% glutaraldehyde in 0.05 M sodium phosphate buffer, pH 7.2, for 2 hr at 4°C, washed in the same buffer without glutaraldehyde for about 2 hr, and dehydrated with a graded ethanol series. Samples were dried in a CPD 020 critical point dryer (Balzers Union; Liechtenstein) and mounted on either ‘Spot-o-glue’ adhesive tabs (Avery; Azusa, CA) or conductive carbon tabs (Ted Pella; Redding, CA) on SEM stabs. The samples were coated with gold-palladium (60:40, w/w) in an Edwards S150B sputter coater (Crawley, England) and examined in a scanning electron microscope (Amray 3300FE; Bedford, MA).

2.3.4 Effects of 2,4-D and Kin

We tested 20 combinations of 2,4-D (0.0, 1.0, 5.0, 25.0, and 125.0 μM) and Kin (0.0, 0.5, 2.5, and 12.5 μM) in the suspension culture medium. Two grams (fresh weight) each of suspension tissue were inoculated into a 50-ml aliquot of liquid medium in a 250-
ml Erlenmeyer flask and incubated for 6 weeks. The cultures were then subjected to the plant regeneration induction process as described previously. Eight replicates (3 flasks each) were used for each of the 20 suspension culture treatments.

2.3.5 Effect of the length of the non-subculture period

Suspension cultures used for this test were continuously incubated in liquid MS medium without being subcultured. Cultures were sampled weekly from Week 4 to Week 9. The samples were subjected to all the steps in our general procedure for plant regeneration.

2.3.6 Effect of the size of cell clusters

Six-week-old suspension cultures were screened through a series of 5 sieves, including mesh 10 (1910 µm), 20 (860 µm), 30 (520 µm), 40 (380 µm), and 50 (280 µm). Each fraction retained on a screen was collected separately. The largest cell aggregates retained on sieve mesh 10 were discarded because preliminary experiments showed this cell size had low regeneration capability. Each collected fraction was inoculated onto an induction medium, as described previously.

2.3.7 Data collection and analysis

In all experiments concerning suspension cultures, 3 plates were used for each replicate and each entire experiment was repeated 2-3 times. The data were expressed as the number of differentiated clumps per gram cells (fresh weight) on the induction media. At the developing stage, 15 to 30 differentiated clumps were transferred to 3 to 6
Magenta vessels containing DM1, 5 clumps in each vessel. We counted the number of differentiated clumps induced per gram tissue and the number of regenerable clumps that developed both shoots and roots or shoots only on the developing medium. The data were subjected to ANOVA and Duncan's Multiple Range Tests at the 5% significance level.

2.4 RESULTS

2.4.1 ESTABLISHMENT AND MAINTENANCE OF SUSPENSION CULTURES

The callus induction rate was investigated 6 weeks after the leaf pieces were placed on the induction medium. Callus induction capabilities of different leaf positions varied greatly different (data not shown). The highest rate of callus induction (>80%) was from the 2-cm basal portions of the 2 innermost leaves. Two types of induced callus (compact and friable) were identified. When both types were inoculated into the liquid medium, only a small number of the friable, callus pieces (~3%) developed into dispersible cell aggregates after 2 to 3 subcultures. Stable suspension cultures were successfully established after 3 to 5 subcultures of the friable callus, and were maintained by subculturing every 3 weeks in the same medium. We were unable to obtain suspension cultures from compact callus.

2.4.2 MORPHOGENESIS OF PLANT REGENERATION

To verify the morphogenic potential of the suspension cultures, cells collected by centrifugation were placed on the induction medium and incubated for up to 6 weeks. The morphogenesis of these cultures was recorded weekly. The initiation and development of the morphogenic events did not occur synchronously. When the
suspension cultures were inoculated onto IM, they appeared as irregular, multicellular masses (Fig. 2-1A). Sizes varied from several to hundreds of cells. A few days to 2 weeks after being placed on the medium, the cell aggregates began to enlarge nonsynchronously and become visually identifiable opaque callus (Fig. 2-1B), which developed from the cell mass and showed some stratified organization of nodule structures under SEM (Fig. 2-2A). At 3-4 weeks after plating, a number of the callus underwent further growth and differentiation into the formation of differentiated clumps (Fig. 2-1C). The SEM examination at this stage revealed that numerous globular, embryo-like structures have developed from the nodules (Fig. 2-2B). Many of these globular structures eventually differentiated into well-organized structures on the differentiated clumps (Fig. 2-1D, 2-1E). These structures included elongated, globular, embryo-like structures and developing shoot apices (Fig. 2-2C). These subsequently developed into either plantlets or shoots (Fig. 2-1F, 2-2D) on the first developing medium containing 1.25 μM BA. Most of the regenerants produced roots when cultured on hormone-free solid media (Fig. 2-1G). The plantlets were transferred to soil in pots and readily acclimatized to greenhouse conditions (Fig. 2-1H).

2.4.3 Effects of 2,4-D and Kin Combinations

Among the various 2,4-D and Kin combinations, the liquid medium containing 5.0 μM 2,4-D and 0.5 μM Kin promoted production of significantly more differentiated clumps (P-value 0.49), which produced single or joined differentiated structures, than the other treatment (Fig. 2-3A). The media containing 0.5 μM Kin generally gave better plant
Fig. 2-1. Morphogenesis process of plant regeneration of *I. germanica* cv. ‘Skating Party’. An irregular, multicellular mass immediately after placement on solid induction medium (Fig. 2-1A). A few days to 2 weeks after being placed on induction medium, the recovered cell aggregates began to enlarge and became visually identifiable opaque callus (Fig. 2-1B). One to 2 weeks later, a number of the callus underwent further growth and differentiation towards the formation of differentiated clumps (Fig. 2-1C), from which numerous well-organized structures were formed (Fig. 2-1D, 2-1E). These structures could initiate plantlets and shoots on the first developing medium (Fig. 2-1F) and the second BA-free developing medium (Fig 2-1G). The plantlets were easily acclimatized under greenhouse conditions (Fig 2-1H).
Fig. 2-2. Scanning electron micrographs of the early stages of plant regeneration from suspension cultures of *Iris germanica*. A. Development of embryo-like nodules, which appeared on visually identifiable opaque callus and organized as strata of cells one on top of the other a few days to 2 weeks after placement on induction medium. B. At 3 to 4 weeks after plating, globular, embryo-like structures developed from the embryo-like nodules 1 to 2 week later. C. Elongated, globular, embryo-like structures and the developing shoot apices. D. A regenerated shoot. Bar sizes: A, B, and C 100 um, D 1 mm.
regeneration for all evaluated concentrations of 2,4-D (Fig. 2-3A). Liquid medium with 5.0 μM 2,4-D consistently produced a high number of regenerable clumps, clumps that survived the transfer from the induction medium to the developing medium and finally produced shoots or plantlets (Fig. 2-3B). Cells grown in the liquid cell-suspension-maintaining medium containing 5.0 μM 2,4-D consistently showed simultaneous development of both shoots and roots on the regenerable clumps on developing medium (Fig. 2-4A). This concentration of 2,4-D (5.0 μM) also enhanced shoot development on the same developing medium (Fig. 2-4B).

2.4.4 EFFECT OF THE LENGTH OF THE NON-SUBCULTURE PERIOD

Cells collected at the sixth week after subculture consistently exhibited both the most differentiated clumps on the induction medium and the most regenerable clumps per gram tissue on the developing medium (Fig. 2-5). Many differentiated clumps derived from the sixth-week cultures survived the transfer from the induction medium to the developing medium, and grew into healthy shoots or plantlets. When cells were collected from cultures maintained in the liquid medium for less than 6 weeks, the numbers of both differentiated and regenerable clumps were dramatically lower and cells from cultures older than 7 weeks showed no regeneration.

2.4.5 EFFECT OF THE SIZE OF CELL CLUSTERS

The numbers of differentiated clumps on the induction medium and regenerable clumps on the developing medium, per gram tissue, were higher with smaller cell clusters or multicellular aggregates (Fig. 2-6). Fractions passing through sieve mesh 30
Fig. 2-3. Effects of plant growth regulator (2,4-D and Kin) combinations in a liquid medium. A. Number of differentiated clumps on an induction medium 5 weeks after inoculation. B. Number of regenerable clumps 5 weeks after being transferred to a solid developing medium.
Fig. 2-4. Effects of 2,4-D and Kin in a liquid medium on differentiation of shoots and roots. A. Number of both shoot- and root-borne regenerable clumps on a solid developing medium 5 weeks after transfer. B. Number of only shoot-borne regenerable clumps 5 weeks after transfer to a solid developing medium.
Number of clumps with shoots only
• g⁻¹ cells

Number of clumps with shoots and roots
• g⁻¹ cells

2,4-D (µM)
- 0.0
- 1.0
- 5.0
- 25.0
- 125.0

Kin (µM)
- 0.0
- 0.5
- 2.5
- 12.5
Fig. 2-5. Effect of the length of the non-subculture period of suspension cultures on plant regeneration.
(<520 \, \mu m \, \text{in diameter}) \, \text{generally produced higher number of both differentiatied and regenerable clumps (Fig. 2-6). The fraction passing through sieve mesh 50 (<280 \, \mu m \, \text{in diameter}) had most differentiated clumps and regenerable clumps per gram tissue.}

2.5 DISCUSSION

Friable callus is usually considered a prerequisite for establishing cell suspension cultures. Lu and Vasil (1981) and Vasil and Vasil (1982), however, established suspension cultures from compact callus of *Panicum maximum* and *Pennisetum americanum*. In the current study we could establish *I. germanica* suspension cultures only from friable callus.

Callus or cell suspension cultures are capable of organogenesis or embryogenesis when first initiated, but they gradually lose morphogenic ability when maintained on a medium that enables continuous growth. This decline or loss may result from nuclear changes (Torrey, 1958; Mitra et al., 1960; Murashige and Nakano, 1965; Torrey, 1967; Smith and Street, 1974) or physiological changes (Syono, 1965; Steward, 1967; Reinert and Backs, 1968; Sussex and Frei, 1968; Reinert et al., 1970). Our suspension cultures from *Iris germanica* cv. ‘Skating Party’, maintained for more than 3 years via repeated subculture, demonstrated a high capacity for regeneration. Furthermore, almost all the regenerated plants showed normal morphology and green color. Among over 500 regenerated plants, we found only 2 to 3 with yellow streaking on the leaves. A few plants were slow growing.

In most previous studies, *in vitro* plant regeneration of iris was assumed to be via somatic embryogenesis (Radojevic et al., 1987; Laublin et al., 1991; Jehan et al., 1994;
Fig. 2-6. Effect of the size of multicellular aggregates from suspension cultures on plant regeneration.
Shimizu et al., 1996, 1997). In a detailed anatomical study of *Iris setosa*, Radojevic and Subotic (1992) demonstrated both somatic embryogenesis and organogenesis. A large proportion of the induced structures developed shoot primodia on the induction medium tested; only a small fraction produced embryo-like structures. Anatomical study during the regeneration process verified the path of plant regeneration from suspension-cultured cells (Wang et al., unpublished.). Plant regeneration from iris suspension-cultured cells was primarily via shoot organogenesis, and also with some somatic embryogenesis. Regardless of the path, the efficiency of regeneration from suspension-cultured cells seemed to be much higher than that of *in vitro* callus culture on agar media as previously reported (Fujino et al., 1972; Meyer et al., 1975; Hussey, 1976; Reuther, 1977; Radojevic et al., 1987; van der Linde and Hol, 1988; van der Linde et al., 1988; Radojevic and Landre, 1990; Laublin et al., 1991; Yabuya et al., 1991; Radojevic and Subotic, 1992; Gozu et al., 1993; Jehan et al., 1994).

Growth regulators exert a most critical influence on plant regeneration. Shimizu et al. (1996) combined 2 mg·l\(^{-1}\) 2,4-D and 0.1 mg·l\(^{-1}\) Kin to initiate friable callus from leaf bases of *I. germanica* cv. 'G1'. However, to establish suspension cultures, 2,4-D was reduced to 1 mg·l\(^{-1}\) and Kin was increased to 1 mg·l\(^{-1}\). A decrease in auxin concentration (2,4-D) with an increase in Kin concentration generally enhance cell growth and the initiation of the proembryogenic cellular aggregates (Kamo et al., 1990; Tsukahara et al., 1996). To maintain the suspension culture, the concentration of 2,4-D is especially important. Vasil (1988) suggested that 1 to 2.5 mg·l\(^{-1}\) of 2,4-D were critical in preserving the embryogenic nature of wheat cultures. Further differentiation of the proembryogenic
callus into mature, regenerated structures occurred only when the 2,4-D was removed from the medium (Fujimura and Komamine, 1980; Kamo et al., 1990).

We also found that plant growth regulators in the liquid suspension culture medium had an important effect on subsequent plant regeneration. Cells from MS liquid medium containing 5.0 μM 2,4-D and 0.5 μM Kin not only produced more differentiated clumps (Fig. 2-3A), but these clumps also had a better survival rate after transfer from the induction medium to the developing medium. They also had a higher tendency to develop into shoots or plantlets (Fig. 2-3B). Cells grown in a liquid medium containing 5.0 μM 2,4-D consistently favored simultaneous development of both shoots and roots on the developing medium (Fig. 2-4A). A ratio of 10:1, 2,4-D: Kin, in the culture medium considerably enhanced regenerable callus formation of iris on agar medium (Laublin et al., 1991; Gozu et al., 1993; Jehan et al., 1994). This was corroborated in the current study, with the best combination being 5.0 μM 2,4-D and 0.5 μM Kin (Fig 2-3) and is consistent with that observed in cereal cultures (Vasil 1988).

Halperin (1966) examined the plant regeneration capability for carrot suspension cultures of different-sized fractions. The 45-75μm fraction, consisted of free cells and proembryogenic masses of various sizes and shapes. These yielded a high percentage of embryos, which were joined in groups. The < 45μm fraction, containing mostly single cells and a small percentage of aggregate units of about 2 to 5 cells, developed a much lower percentage of embryos. Though the exact mechanism is unknown, we can concluded that the size of multicellular aggregates in suspension culture is an important factor affecting plant regeneration efficiency.
Regeneration rates of clumps > 520 μm (remaining above sieve mesh 30) were very low; rates of clumps < 380 μm (passing sieve mesh 30) were progressively higher. The highest rate occurred for clump mixtures < 280 μm (passing through sieve mesh 50). Cells in smaller-sized clumps generally had very dense cytoplasms resembling embryogenic cell lines in other species (Halperin, 1966). The larger the clumps, the more difficult it may have been for the majority of the cells to respond to the inductive stimuli for morphogenesis. Therefore, the physiological state of the larger clumps might not have been suitable for regeneration. In contrast, clumps < 190 μm (passing through sieve mesh 80) also demonstrated very low regeneration (data not shown). In suspension culture of carrots, cells needed to form primary differentiating states of proembryogenic masses for further differentiation on an induction medium (Halperin, 1966). If the cell aggregate clumps were too small, they could not reach the required mass and regeneration could not proceed. To some extent the cells, which initially establish polarity, arise because of metabolic gradients from the center to the periphery of the proembryogenic cell mass. Even in the appropriate physiological (regeneration competent) state, only limited numbers of single cells can accommodate the changes if dissociated from the cell mass.

The interval for subculturing of suspension cultures depends on plant genotypes, and usually ranges from 1 week to 4 weeks (Kamo et al., 1990; Wang and Nguyen, 1990; Shimizu et al., 1996). Almost all reports on regeneration from suspension-cultured cells have focused on the type and concentrations of growth regulators, medium compositions and concentrations, and cultural conditions. There have been no reports on the influence of the length of the non-subculture period on regeneration efficiency. The most important
finding in our current study may be that the length of the couture period had a remarkable effect on plant regeneration in *Iris germanica*. The subculture interval for regular maintenance was 3 weeks. If extended to 5 weeks, most cells or cell aggregates in the suspension cultures became necrotic soon after they were transferred to the fresh liquid medium. If, however, the cultures were held intact in the liquid medium for 6 to 7 weeks without subculturing, they were still recoverable and gave rise to the highest regeneration when transferred to the induction medium. Beyond this period, both recovery and regeneration rates were sharply reduced. As the period varied from the last subculture, the suspension cultures may have represented different physiological states closely associated with regeneration competency of the cell suspension cultures. The reason for such a difference is currently unknown; and further study is needed on understanding the mechanism of the aging effect on plant regeneration of *Iris*.

In the current study, several parameters were optimized in suspension cultures for efficient plant regeneration, but the induction medium used still might not be ideal. The parameters in the induction phase must also be optimized to further improve regeneration efficiency.

2.6 LITERATURE CITED


CHAPTER 3

OPTIMIZATION OF CONDITIONS FOR THE INDUCTION OF PLANT REGENERATION FROM SUSPENSION-CULTURED CELLS OF IRIS GERMANICA CV. ‘SKATING PARTY’

3.1 ABSTRACT

A stable suspension culture of Iris germanica was established previously (Wang, in prep.) and factors that affect plant regeneration at the suspension culture phase were optimized. The objective of the current study was to further improve the efficiency of plant regeneration at the induction and developing phases. The influence of the combinations of Kin and NAA on the induction of regenerable clumps and the subsequent development of induced structures were tested. The highest regeneration rate (66.7%) and the rate of regenerable clumps that simultaneously developed both shoots and roots (>63%) was consistently observed in combinations of either 2.5 or 12.5 μM Kin and 0.5 μM NAA for 6 weeks. The developing medium containing 1.25 μM N⁶-benzyladenine (BA) was optimal for regeneration and the percentage of plantlets developing shoots and roots simultaneously. Rooted plantlets were easily acclimatized, transplanted to various soil mixtures, and grown in the greenhouse.

3.2 INTRODUCTION

Plant regeneration in vitro via callus culture is reported for a number of Iris species, including I. germanica (Fujino et al., 1972; Meyer et al., 1975; Hussey, 1976; Reuther, 1977; Radojevic et al., 1987; van der Linde and Hol, 1988; van der Linde et al., 1988; Radojevic and Landre, 1990; Laublin et al., 1991; Yabuya et al., 1991; Radojevic
and Subotic, 1992; Gozu et al., 1993; Jehan et al., 1994). In addition, *I. germanica* were regenerated from protoplast cultures (Shimizu et al., 1996) and suspension cultures (Shimizu et al., 1997). Nevertheless, low efficiency in regeneration has hindered genetic transformation of this species.

An efficient and reproducible protocol for plant regeneration from cell suspension cultures of *I. germanica* cv. ‘Skating Party’ has been developed (Wang, in prep.). Several parameters, critical in the cell suspension culture phase, were optimized, including concentrations of plant growth regulators, size of cell aggregates, and the length of the non-transferred suspension culture period. However, the conditions for inducing plant regeneration might not be optimal. In the current study, factors affecting plant regeneration in the induction and development steps were examined to further improve the efficiency of plant regeneration for *I. germanica*. This system is currently being used to develop a reliable genetic transformation protocol for iris.

### 3.3 MATERIALS AND METHODS

#### 3.3.1 MEDIA

Suspension cultures of *I. germanica* cv. ‘Skating Party’ were maintained in liquid MS basal medium (Murashige and Skoog, 1962), supplemented with 290 mg·l\(^{-1}\) proline, 5 \(\mu\text{M}\) 2,4-D, and 1.0 \(\mu\text{M}\) Kin. The induction medium (in which suspension-cultured cells differentiate into regenerable structures) was MS basal medium with 250 mg·l\(^{-1}\) proline, 250 mg·l\(^{-1}\) casein hydrolysate, 10 mg·l\(^{-1}\) pantothenic acid, 4.5 mg·l\(^{-1}\) niacin, 1.9 mg·l\(^{-1}\) thiamin, 50 g·l\(^{-1}\) sucrose, and 2.0 g·l\(^{-1}\) Phytagel\(^\text{TM}\) (Sigma, St. Louis, MO). The pH was adjusted to 5.7 before autoclaving. The first developing medium, DM1 (for shoot
development from the regenerable structures) and the second developing medium, DM2 (primarily for root development from regenerated shoots to form normal plantlets) were the same as the induction medium except for the addition of growth regulators (see section 3.3.3). All media were autoclaved for 25 min at 120°C (15 psi).

3.3.2 Preparation of Suspension Cultures for Regeneration Experiments

Stock suspension cultures were maintained in a liquid medium in the dark on a gyrating shaker (100 rpm, 23°C), with regular subculture every 3 weeks. For regeneration experiment, six-week-old cultures were screened through a sieve mesh 30. The pass-through fraction (< 520 μm) was collected in 50-ml tubes and centrifuged at 2000 rpm for 10 min in a clinical centrifuge (IEC, Model HN-SII; International Equipment Co., Needham Heights, Mass.). The pelleted cells were weighed after the liquid was discarded and were then resuspended in a liquid induction medium to a density of 0.2 gm-1. A 0.5-ml aliquot was inoculated onto a 15- x 60-mm plastic petri dish containing 20 ml of the solid induction medium. The cultures were then incubated in the dark at 25°C.

3.3.3 Effect of Kin and NAA Combinations in Induction Medium

Sixteen combinations (3 plates each) of NAA (0.0, 0.5, 2.5, and 12.5 μM) and Kin (0.0, 2.5, 12.5, and 62.5 μM) in the solid induction media were evaluated for their effects on plant regeneration. After the cultures were incubated at 25°C in the dark for 5 weeks, the number of differentiated clumps and their quality (size constituents) were recorded. The differentiated clumps, from which numerous white structures originated, developed
from individual cell aggregates of the suspension culture. The experiment was repeated 3 times.

To assess the response of differentiated clumps from each growth regulator combination, the clumps were randomly sampled and transferred to DM1 with 1.25 μM BA in Magenta GA-7 vessels (Sigma). The clumps were incubated for 6 weeks under light (50 μE) at 23°C. The number of regenerable clumps (sum of the differentiated clumps that developed shoots only, and both shoots and roots) and the types of their development (the clumps developed shoots only, roots only or both shoot and roots) were recorded. The regenerable clumps were then transferred to the second developing medium DM2 (no growth regulators).

3.3.4 Effect of BA Concentrations on Development of Differentiated Clumps

Three concentrations of BA (0.0, 1.25, and 2.5 μM) were evaluated in DM1 for their effect on the further differentiation of regenerable clumps. Clumps from the combination of 2.5 μM Kin and 0.5 μM NAA induction agar medium, which demonstrated the highest % plant regeneration and most desirable developmental orientation (see section 3.4.2), were randomly sampled and transferred to each of the three DM1 with different concentration of BA and incubated for 6 weeks under light (50 μE) at 23°C.
3.3.5 RELATIONSHIP BETWEEN SIZE AND AGE OF DIFFERENTIATED CLUMPS AND THEIR REGENERATION CAPABILITY

To determine the optimal period for clumps to remain on induction medium, the screened cells were inoculated onto IM containing 2.5 μM Kin and 0.5 μM of NAA. The differentiated clumps were classified into 4 sizes at week 5 (large: >10 mm; medium: ~5 to 10 mm; small: ~2 to 5 mm and very small: < 2 mm). The clumps from each size class were randomly divided into 5 groups and the number of clumps in each size class were recorded. The first group from each of the 4 size classes were remained on the original IM in dark to allow for continuous growth and differentiation. The other 4 groups (45 clumps each, which were randomly assigned to three replicates) for each of the 4 size classes were transferred to DM1 with 1.25 μM in the vessels from the 6th to the 9th week, respectively, and incubated under light (50 μE) at 23°C; The size distribution of the differentiated clumps in the first group were monitored and recorded weekly from week 6 to week 9. The development of the differentiated clumps (shoots only, roots only or both shoots and roots) in the other 4 groups was recorded 5 week after each transfer.

3.3.6 ACCLIMATIZATION AND SUBSTRATES FOR GROWING IN VITRO REGENERATED IRIS PLANTS IN THE GREENHOUSE

Rooted plantlets cultured for 6 weeks on the DM2 were transferred to the greenhouse for acclimatization under misting or non-misting conditions. The 8 substrates tested included peat moss only; perlite only; sandy loam; 1:1 peat moss, sandy loam (v/v); 1:1 peat moss, perlite (v/v); 1:1 perlite, sandy loam (v/v); 1:1:1 peat moss, perlite, sandy loam (v/v/v); and 1:1:1 peat moss, pumice, sandy loam (v/v/v). Five plantlets each were placed in 8 6"-plastic pots (Anderson Die & MFG. Co., Portland, OR) filled with one of the substrates. The 8 pots for each substrate were divided into 2 groups: one group was maintained on the misting bench, the other on a non-misting bench. The relative humidity (RH) on the misting bench was close to 100% (misting at 1 min intervals.); RH
on the non-misting bench was ~ 60 to 80%. The plantlets on the non-misted bench were watered every other day. All plants were fertilized monthly with Nutricode type100, a controlled-release fertilizer (Fertilizer Co. LTD; Tokyo, Japanese.). Temperature was maintained at 70 ± 5°F. After 6 weeks the misting-bench plants were transferred to a non-misting bench. Percentage of survival, fresh weights of whole plants, shoots, and roots only were measured after 4 months.

3.4 RESULTS

3.4.1 EFFECTS OF KIN AND NAA COMBINATIONS IN INDUCTION MEDIUM

After 2 weeks of incubation on induction medium, some white structures began to appear on the differentiated clumps. By week 6, differentiated clumps were observed from all 16 Kin and NAA combinations. The number of differentiated clumps induced differed significantly among the combinations of Kin and NAA (Fig. 3-1). ANOVA analysis demonstrated the significant effects of Kin (p-value 0.0001) and NAA (p-value 0.0001), and their interaction (p-value 0.0541). The lower concentrations of NAA and Kin gave rise to more clumps per gram of suspension cells (Fig 3-1). The constituents of the differentiated clumps differed significantly among the 16 treatments (all p-values < 0.01). The highest proportion of large clumps (diameter ≥ 1.0 cm) were derived from 0.5 μM NAA with either 2.5 μM Kin (8.3% ± 0.31%) or 12.5 μM Kin (12.9% ± 0.5%).
Fig. 3-1. Effect of Kin and NAA combinations in IM on the quantity of differentiated clumps. After 6 weeks on IM, the recovered suspension cells differentiated into callus clumps on which white structures were born. Some of the structures are regenerable after being transferred to DM1 and DM2.
3.4.2 Effects of KIN and NAA Combinations on the Subsequent Development of Regenerable Clumps

Kin and NAA in induction medium affected not only the number of differentiated clumps induced, but also the subsequent development of the regenerable clumps, which include both the % regenerable clumps and the frequency of clumps that simultaneously developed both shoots and roots on the DM1 with 1.25 μM BA (Table 3-1). The highest % regeneration and the most clumps that produced both shoots and roots were observed in the combinations of 0.5 μM NAA with either 2.5 or 12.5 μM Kin.

3.4.3 Effect of BA Concentrations on the Development of Differentiated Clumps

Clumps on the DM1 containing 1.25 μM BA produced both a high rate of plant regeneration and the most desirable developmental clumps (Table 3-2). Although DM1 containing 2.5 μM BA had more regeneration, the rate of the desirable developmental clumps (with both shoots and roots) was very low. A large proportion of the regenerable clumps developed only shoots on this medium.

After being transferred to the DM2 (no growth regulator), the majority of the clumps recovered well from the transfer shock. Clumps from the DM1 containing either 0.0 or 1.25 μM BA subsequently developed healthy plantlets with both shoots and roots (Fig. 3-2A); The majority of the clumps from DM1 containing 2.5 μM BA showed poor root development or no roots at all (Fig. 3-2B).

To assess the effect of BA concentration in DM1 on the size of plantlets, the mean percentage of clumps that developed different sizes of shoots was compared. Compared to 23.2 (± 1.6%) for DM1 without BA and 71.7% (± 5.0%) for DM1 with 2.5 μM BA
Table 3-1. Effects of Kin and NAA combinations in IM on the regeneration and development of differentiated clumps in terms of % regeneration\(^x\) and % development during the 6-week subsequent culture on DM1 with 1.25 μM BA.

<table>
<thead>
<tr>
<th>Growth regulators (μM)</th>
<th>No. of clumps tested</th>
<th>% Regeneration(^x)</th>
<th>% Development</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Shoots and roots</td>
<td>Shoots only</td>
<td>Roots only</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kin</td>
<td>NAA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td>0.0</td>
<td>54</td>
<td>12.3 d(^y)</td>
<td>12.3 d</td>
<td>0.0 a</td>
<td>5.6 bc</td>
</tr>
<tr>
<td>0.5</td>
<td>45</td>
<td>45.1 abc</td>
<td>3.3 abc</td>
<td>1.9 a</td>
<td>4.4 bc</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>36</td>
<td>52.8 abc</td>
<td>44.4 abc</td>
<td>8.3 a</td>
<td>8.3 bc</td>
<td></td>
</tr>
<tr>
<td>12.5</td>
<td>36</td>
<td>36.1 abcd</td>
<td>33.3 bcd</td>
<td>2.8 a</td>
<td>36.1 a</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>0.0</td>
<td>36</td>
<td>12.3 d</td>
<td>10.5 d</td>
<td>1.8 a</td>
<td>27.8 ab</td>
</tr>
<tr>
<td>0.5</td>
<td>63</td>
<td>66.7 a</td>
<td>64.8 a</td>
<td>1.9 a</td>
<td>11.1 bc</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>45</td>
<td>50.0 abc</td>
<td>50.0 abc</td>
<td>0.0 a</td>
<td>20.0 ab</td>
<td></td>
</tr>
<tr>
<td>12.5</td>
<td>36</td>
<td>33.3 bcd</td>
<td>33.3 bcd</td>
<td>0.0 a</td>
<td>38.9 a</td>
<td></td>
</tr>
<tr>
<td>12.5</td>
<td>0.0</td>
<td>36</td>
<td>26.7 cd</td>
<td>22.2 cd</td>
<td>4.4 a</td>
<td>13.9 bc</td>
</tr>
<tr>
<td>0.5</td>
<td>36</td>
<td>66.7 a</td>
<td>63.9 a</td>
<td>2.8 a</td>
<td>0.0 d</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>36</td>
<td>52.8 abc</td>
<td>44.4 abc</td>
<td>8.3 a</td>
<td>8.3 bc</td>
<td></td>
</tr>
<tr>
<td>12.5</td>
<td>36</td>
<td>27.8 cd</td>
<td>25.0 cd</td>
<td>2.8 a</td>
<td>22.2 ab</td>
<td></td>
</tr>
<tr>
<td>62.5</td>
<td>0.0</td>
<td>36</td>
<td>38.9 abcd</td>
<td>33.3 bcd</td>
<td>5.6 a</td>
<td>13.9 bc</td>
</tr>
<tr>
<td>0.5</td>
<td>27</td>
<td>44.4 abc</td>
<td>38.9 abcd</td>
<td>5.6 a</td>
<td>0.0 d</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>36</td>
<td>63.9 ab</td>
<td>55.5 ab</td>
<td>8.3 a</td>
<td>5.6 bc</td>
<td></td>
</tr>
<tr>
<td>12.5</td>
<td>45</td>
<td>33.3 bcd</td>
<td>30.6 bcd</td>
<td>2.8 a</td>
<td>8.9 bc</td>
<td></td>
</tr>
</tbody>
</table>

\(^x\) Percentage of clumps which could develop regenerable organs (both shoots and roots, or shoots only).

\(^y\) Duncan's Multiple Range Test for variables at alpha = 0.05 level. Means with the same letter are not significantly different.
Table 3-2. Effects of BA concentration in the first developing medium on the regeneration and developmental orientation of the differentiated clumps.

<table>
<thead>
<tr>
<th>BA (µM)</th>
<th>No. of clumps tested</th>
<th>% Regeneration$^x$</th>
<th>% Large shoots$^y$</th>
<th>% Development</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Shoots and roots</td>
<td>Shoots only</td>
<td>Roots only</td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td>63</td>
<td>54.0 a$^z$</td>
<td>23.2 c</td>
<td>47.6 ab</td>
<td>6.3 b</td>
<td>11.1 a</td>
<td></td>
</tr>
<tr>
<td>1.25</td>
<td>63</td>
<td>66.7 a</td>
<td>81.8 a</td>
<td>64.8 a</td>
<td>1.9 b</td>
<td>11.1 a</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>45</td>
<td>68.9 a</td>
<td>71.7 b</td>
<td>37.8 b</td>
<td>31.1 a</td>
<td>6.7 a</td>
<td></td>
</tr>
</tbody>
</table>

$^x$Percentage of clumps which could develop regenerable organs (both shoots and roots, or shoots only).

$^y$The differentiated clumps were produced on an induction medium containing 2.5 µM Kin and 0.5 µM NAA.

$^z$Duncan's Multiple Range Test for variables at alpha = 0.05 level. Means with the same letter are not significantly different.
Fig. 3-2. Effect of BA in DM1 on development of differentiated clump induced from suspension cells on IM with 2.5 μM Kin and 0.5 μM NAA for 6 weeks. After 4 weeks, A) medium containing 1.25 μM BA showed well-developed shoots and roots; B) medium containing 2.5 μM BA showed poor root development from clumps.
3.4.4 RELATIONSHIP BETWEEN SIZE AND AGE OF DIFFERENTIATED CLUMPS AND THEIR REGENERATION CAPABILITY

Clumps were arbitrarily divided into 4 size classes (Fig. 3-3). The changes in the proportion of each class were monitored from week 6 to week 9. Each type of clump, except for the large clumps, underwent a continuous transition from very small to large (Table 3-3). The proportion of the smaller size class decreased while the big clumps cumulatively increased.

After the induced clumps were incubated for 5 weeks on a developing medium containing 1.25 µM BA, the % regenerable clumps (Table 3-4) and their capability of developing into large plants were investigated. Each of the 4 clump sizes had its highest regeneration if the clumps were transferred to the developing medium at week 6. At a given age, bigger clumps had higher ability of regeneration and growth and tended to simultaneously develop both shoots and roots (Fig. 3-4). A prolonged non-subculture period on the induction medium produced a lower regeneration rate and resulted in a reduction of clumps with both shoots and roots (Fig. 3-4).

The size of the shoots that developed from induced differentiated clumps on the developing medium was used to characterize the quality of the regenerable clumps. Both age (the period the culture remained on the induction medium) and size of the differentiated clumps determined the capability for shoot development. Younger and larger clumps produced a higher proportion of shoots > 3cm in height (Table 3-4).
Fig. 3-3. Size classification of differentiated clumps developed on IM 5 weeks after suspension cells were inoculated. Size classes are showed from bottom to top: large = > 10 mm; Medium = ~5 to 10 mm; Small = ~ 2 to 5 mm; Very Small = < 2 mm.
Table 3-3. Size transition of differentiated clumps between classes on IM from week 6 through week 9.

<table>
<thead>
<tr>
<th>Age of clumps (weeks)</th>
<th>% of each class</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Large(^y)</td>
</tr>
<tr>
<td>6</td>
<td>29.2(^z)</td>
</tr>
<tr>
<td>7</td>
<td>47.6</td>
</tr>
<tr>
<td>8</td>
<td>78.1</td>
</tr>
<tr>
<td>9</td>
<td>85.7</td>
</tr>
</tbody>
</table>

\(^y\)Large: > 10mm; Medium: ~5 to 10 mm; Small: ~2 to 5 mm; Very Small: < 2 mm.

\(^z\)Means based on continuous observations on the samples assigned at week 5.
Fig. 3-4. Difference in regeneration and development among size classes of the differentiated clumps 5 weeks after being transferred onto DM1. Size classes of differentiated clumps: A = large, B = medium, C = small, D = very small.
Table 3-4. Relationship between age and size\textsuperscript{w} of differentiated clumps\textsuperscript{x}, in terms of % regeneration and capability of developing big shoots\textsuperscript{y}.

<table>
<thead>
<tr>
<th>Age of clumps (weeks)</th>
<th>% Regeneration</th>
<th>% clumps producing big shoots</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Big</td>
<td>Medium</td>
</tr>
<tr>
<td>6</td>
<td>100.0 a\textsuperscript{z}</td>
<td>95.0 a</td>
</tr>
<tr>
<td>7</td>
<td>100.0 a</td>
<td>93.3 a</td>
</tr>
<tr>
<td>8</td>
<td>100.0 a</td>
<td>73.4 b</td>
</tr>
<tr>
<td>9</td>
<td>97.8 a</td>
<td>53.8 c</td>
</tr>
</tbody>
</table>

\textsuperscript{w}See footnote in Table 3-3.
\textsuperscript{x}Clumps were derived from an induction medium containing 2.5 µM Kin and 0.5 µM NAA, then transferred to a developing medium containing 1.25 µM BA.
\textsuperscript{y}Big shoots were defined as > 3 cm in height.
\textsuperscript{z}Duncan’s Multiple Range Test for variables at alpha = 0.05 level. Means with the same letter are not significantly different.
3.4.5 Effects of acclimatization conditions and substrates on plant establishment in the greenhouse

Plant recovery (p-value ~0.01) and growth (p-value < 0.01) after transferring from in vitro culture to greenhouse cultivation varied greatly among substrates (Table 3-5). However, the difference in the effect between misting and non-misting on plant recovery was not significant (all p-values > 0.35, data not show). Therefore, the data from misting and non-misting conditions were combined for statistical analysis of substrates for greenhouse cultivation. Except two poor substrates, peat alone and perlite alone, which resulted in fewer recovery and less growth, the other six substrates showed no significant differences either for recovery or for fresh weight of total plant, shoots or roots only. Nevertheless, the substrates of 1:1 peat moss, perlite, 1:1:1 peat moss, perlite, sandy loam and 1:1:1 peat moss, pumice, sandy loam gave rise to the highest recovery and plant growth.

3.5 DISCUSSION

The combination of auxin and cytokinin in media is one of most important factors for in vitro plant regeneration in Iris cultures (Radojevic et al., 1987; Laublin et al., 1991; Radojevic and Subotic, 1992; Gozu et al., 1993; Jehan et al., 1994; Shimizu et al., 1996). The most effective auxin for inducing embryogenic callus is usually 2,4-D when combined with Kin. Similar results are found in regeneration from cell suspension cultures of rice (Oryza sativa L) (Inoue and Maeda, 1980; Ling et al., 1983; Ozawa and Komamine, 1989; Tsukahara and Hirosawa, 1992; Tsukahara et al., 1996). Suspension cultures transferred from high 2,4-D- to low or no 2,4-D medium readily regenerate (Smith and Street, 1974; Fujimura and Komamine, 1980). However, 2,4-D in a liquid
Table 3-5. Effect of substrates of greenhouse cultivation on plant recovery from transfer and acclimatization, and on growth in fresh weight after 6 month’s cultivation in greenhouse.

<table>
<thead>
<tr>
<th>Tested Substrates</th>
<th>% survival</th>
<th>Plant fresh weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>Peat</td>
<td>50.0</td>
<td>b</td>
</tr>
<tr>
<td>Perlite</td>
<td>57.5</td>
<td>b</td>
</tr>
<tr>
<td>Sandy loam</td>
<td>85.0</td>
<td>a</td>
</tr>
<tr>
<td>Peat : perlite</td>
<td>85.0</td>
<td>a</td>
</tr>
<tr>
<td>Peat : sand loam</td>
<td>70.0</td>
<td>ab</td>
</tr>
<tr>
<td>Perlite : sand</td>
<td>87.5</td>
<td>a</td>
</tr>
<tr>
<td>Peat : Perlite : Sandy loam</td>
<td>82.5</td>
<td>a</td>
</tr>
<tr>
<td>Peat : pumice : Sandy loam</td>
<td>90.0</td>
<td>a</td>
</tr>
</tbody>
</table>

P-value for Substrates

| P-value | <0.00 | < 0.01 | < 0.05 | < 0.001 |

\(^z\) Duncan’s Multiple Range Test for variables at alpha = 0.05 level. Means with the same letter are not significantly different. Values followed by the same letter in a single column are not significant different.
medium is essential for suspension cultures to grow continuously and form proembryogenic or proorganogenic masses. Plants can be regenerated from these masses when 2,4-D is reduced or eliminated from the medium (Halperin, 1966).

Although 2,4-D inhibits plantlet development from callus, NAA in combination with Kin promotes plant regeneration. In the current study, several combinations of NAA and Kin concentrations were tested to improve the efficiency of plant regeneration in iris cultures. Low concentrations of NAA and Kin in the induction media produced a higher number of good quality, regenerable clumps.

There may also have been a weak interaction between Kin and NAA on the number of induced differentiated clumps. For instance, the number of clumps from the induction medium containing 2.5 μM Kin was significantly higher than from that containing 0.0 μM Kin, both at 0.0 μM NAA. More differentiated clumps also were produced at 2.5 and 12.5 μM Kin than at 0.0 μM Kin, when 12.5 μM NAA was in the induction medium (Fig. 3-1).

The combinations of plant growth regulators that produced the most differentiated clumps from suspension cells did not give rise to the highest number of regenerable clumps. The reason may be that the growth, differentiation and subsequent development of cells or tissues preliminarily determined by the balance of endogenous hormonal substances (Salisbury and Ross, 1992), which affected by the addition of exogenous hormonal substances in medium. In this thesis research, low concentrations of both Kin and NAA were desired for the induction events but not for further development of the
induced structures. Regeneration required 0.5 µM NAA combined with 2.5 or 12.5 µM Kin in the induction medium.

BA in the first developing medium exerted a substantial influence on the development of clumps. Induction medium containing 2.5 or 12.5 µM Kin and 0.5 µM NAA produced the highest regeneration efficiency (both regeneration rate and % differentiated clumps that developed both shoots and roots) on the first developing medium containing 1.25 µM BA (Table 3-1). Regeneration efficiency was slightly lower on the second developing medium without addition of BA. A higher concentration of BA (2.5 µM) enhanced shoot development but inhibited simultaneous rooting. This was similar to the response in the regeneration of *Cicer arietinum* (Polisetty et al., 1997).

A prolonged incubation period (beyond 6 weeks) on the induction medium decreased both the quantity and quality of the differentiated clumps. Clumps that were kept on the induction medium for longer than 6 weeks showed a transition to a large size class no matter what their original size was. The 6-week-old clumps of all sizes had the highest physiological capability for regeneration and development.

### 3.6 LITERATURE CITED


CHAPTER 4

CONCLUSION

The optimal conditions for efficient *in vitro* plant regeneration from suspension cultured cells of *Iris* include: 1) growing suspension cultures in a liquid medium containing 5 μM 2,4-D and 0.5 μM Kin for 6 weeks. The suspension cultures are then passed through a stainless sieve to select cell clusters with diameters < 280 μm. 2) The screened cell fraction is then inoculated onto an induction medium containing 2.5 to 12.5 μM Kin and 0.5 μM NAA for 6 weeks. 3) The differentiated clumps are transferred to a developing medium containing 1.25 μM BA for 5 to 6 weeks. 4) The regenerable clumps are then transferred to a BA-free developing medium for root development. 5) The rooted plantlets are transplanted to a substrate containing 1:1:1 peat moss : perlite : sandy loam (v/v/v). Under these conditions, up to 8000 plantlets can been regenerated from one gram of screened cells in about 12 to 15 weeks.
BIBLIOGRAPHY


