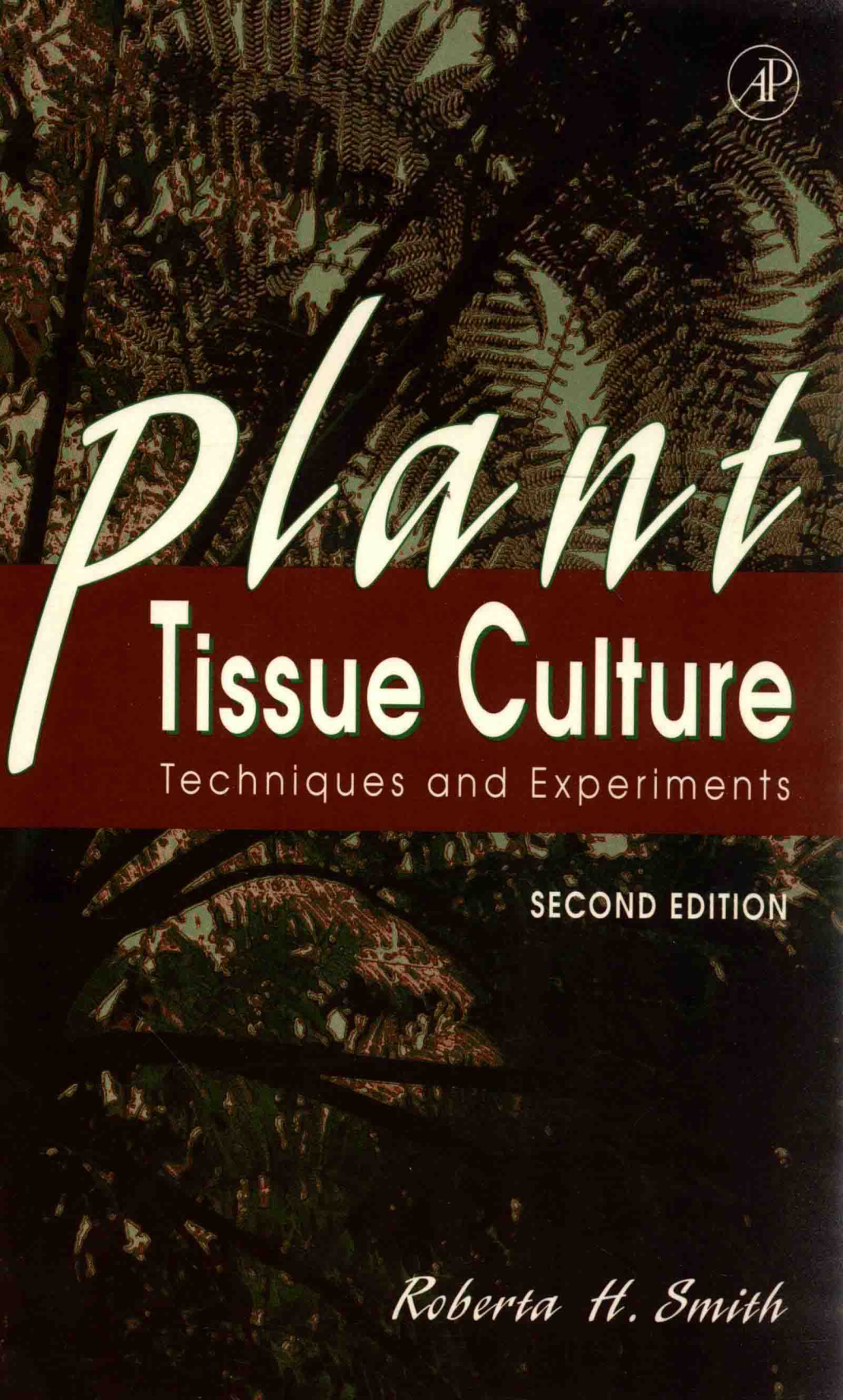
plant
Tissue Culture
Techniques and Experiments
SECOND EDITION
Roberta H. Smith

PLANT TISSUE CULTURE

TECHNIQUES AND EXPERIMENTS

SECOND EDITION

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PREFACE

This manual resulted from the need for plant tissue culture laboratory exercises that demonstrate major concepts and that use plant material that is available year round. The strategy in developing this manual was to devise exercises that do not require maintenance of an extensive collection of plant materials, yet give the student the opportunity to work on a wide array of plant materials.

The students who have used these exercises range from high school (science fair and 4-H projects) to undergraduate and graduate levels. The manual is predominately directed at students who are in upper-level college or university classes and who have taken courses in chemistry, plant anatomy, and plant physiology.

Before starting the exercises, students should examine Chapters 2 through 5, which deal with the setup of a tissue culture laboratory, media preparation, explants, aseptic technique, and contamination. The information in these chapters will be needed in the exercises that follow.

The brief introduction to each chapter is not intended to be a review of the chapter's topic but rather to complement lecture discussions of the topic. In this revised edition, Dr. Trevor Thorpe has contributed a chapter on the history of plant cell culture. Dr. Brent McCown contributed a chapter on woody trees and shrubs.

In many instances, plant material initiated in one exercise is used in subsequent exercises. Refer to Scheduling and Interrelationships of Exercises to obtain information on the time required to complete the exercises and how they relate to one another.

All of the exercises have been successfully accomplished for at least 10 semesters. Tissue culture, however, is still sometimes more art than science, and variation in individual exercises can be expected.

Roberta H. Smith

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The contributions of Dr. Trevor Thorpe with the chapter on the history of plant cell culture and Dr. Brent McCown with a chapter on woody trees and shrubs are greatly appreciated.

Last, I thank my husband, daughter Christine, and son Will, for their support of this project.

SCHEDULING AND INTERRELATIONSHIPS OF EXERCISES

I. Aseptic Germination of Seed (Chapter 4)
   Carrot: 1–2 weeks; Cotton, Sunflower: 1 week
   a. Callus Induction (Chapter 6): 6 weeks
      Broccoli, Lemon 6–8 weeks
      Carrot: 2 subcultures, 6 weeks each = 3 months
      1. Salt Selection *in Vitro* (Chapter 6): 4 weeks
      2. Suspension Culture (Chapter 7): 2 weeks
         Carrot
         a. Somatic Embryogenesis (Chapter 7): 3–4 weeks
         b. Explant Orientation (Chapter 6): 6 weeks
            Cotton: 2 subcultures, 6 weeks each
      1. Protoplast (Chapter 13): 2 days
      2. Cellular Variation (Chapter 6): 4 weeks
      3. Growth Curves (Chapter 6): 6 weeks

II. Tobacco Seed Germination (Chapter 6): 3 weeks
   a. Callus Induction (Chapter 6): 2 subcultures, 6 weeks each

III. Establishment of Competent Cereal Cell Cultures (Chapter 6): 2–3 weeks
   a. Rice Subculture (Chapter 7): 3 weeks
      1. Plant Regeneration: 4–6 weeks

IV. Potato Shoot Initiation (Chapter 7): 6 weeks
   a. Potato Tuberization (Chapter 7): 4–6 weeks

V. Douglas Fir Seed Germination (Chapter 4): 2–4 weeks
   a. Primary Morphogenesis (Chapter 7): 4 weeks

VI. Petunia/Tobacco Leaf Disk Transformation (Chapter 14): 6 weeks

VII. Petunia Shoot Apex Transformation (Chapter 14): 4–6 weeks

VIII. Solitary Exercises
- a. Bulb Scale Dormancy (Chapter 7): 6–8 weeks
- b. Datura Anther Culture (Chapter 9): 4–8 weeks; 10 weeks to obtain flowering plants
- c. African Violet Anther Culture (Chapter 9): 7–8 weeks
- d. Tobacco Anther Culture (Chapter 9): 7–8 weeks; 2–3 months to obtain flowering plants
- e. Corn Embryo Culture (Chapter 10): 72 hr
- f. Crabapple and Pear Embryo Culture (Chapter 10): 2–3 weeks
- g. Shoot Apical Meristem (Chapter 11): 4–6 weeks
- h. Diffenbachia Meristem (Chapter 11): 4–6 weeks
- i. Garlic Propagation (Chapter 11): 4 weeks
- j. Boston Fern Propagation (Chapter 12)
  Stage I: 6–8 weeks
  Stage II: 4–6 weeks
  Stage III: 2–3 weeks
- k. Staghorn Fern Propagation (Chapter 12)
  Stage I: 2–3 weeks
  Stage II: 6 weeks
  Stage III: 4–6 weeks
- l. Ficus Propagation (Chapter 12)
  Stage I: 4–6 weeks
  Stage II: 4–6 weeks
  Stage III: 4 weeks
- m. Kalanchoe Propagation, Stages I & II (Chapter 12): 4 weeks
- n. African Violet, Stages I & II (Chapter 12): 4 weeks
- o. Pitcher Plant, Stages I & II (Chapter 12): 6 weeks
- p. Cactus Propagation (Chapter 12)
  Stage I: 4–6 weeks
  Stage II: 4–6 weeks
  Stage III: 8 weeks
- q. Rhododendrons and Azaleas (Chapter 8): 4–6 weeks
- r. Birch Trees (Chapter 8): 2 weeks seed germination: 4–6 weeks
- s. White Cedar (Chapter 8): 4–6 weeks
- t. Roses (Chapter 8): 4–6 weeks

CONTENTS

CHAPTER 1

HISTORY OF PLANT CELL CULTURE

Trevor A. Thorpe
The University of Calgary

INTRODUCTION

Plant cell/tissue culture, also referred to as *in vitro*, axenic, or sterile culture is an important tool in both basic and applied studies as well as in commercial application (see Thorpe, 1990). Although Street (1977) has recommended a more restricted use of the term, plant tissue culture is generally used for the aseptic culture of cells, tissues, organs, and their components under defined physical and chemical conditions *in vitro*. Perhaps the earliest step toward plant tissue culture was made by Henri-Louis Duhumel du Monceau in 1756, who, during his pioneering studies on wound-healing in plants, observed callus formation (Gautheret, 1985). Extensive microscopic studies led to the independent and almost simultaneous development of the cell theory by Schleiden (1838) and Schwann (1839). This theory holds that the cell is the unit of structure and function in an organism and therefore capable of autonomy. This idea was tested by several researchers, but the work of Vöchting (1878) on callus formation and on the limits to divisibility of plant segments was perhaps the most important. He showed that the upper part of a stem segment always produced buds and the lower end callus or roots independent of the size until a very thin segment was reached. He demonstrated polar development and recognized that it was a function of the cells and their location relative to the cut ends.

The theoretical basis for plant tissue culture was proposed by Gottlieb Haberlandt in his address to the German Academy of Science in 1902 on his experiments on the culture of single cells (Haberlandt, 1902). He opined that to "my knowledge, no systematically organized attempts to culture isolated vegetative cells from higher plants have been made. Yet the results of such culture experiments should give some interesting insight to the properties and potentialities which the cell as an elementary organism possesses. Moreover, it would provide information about the inter-relationships and complementary influences to which cells within a multicellular whole organism are exposed" (from the English translation by Krikorian &

Berquam, 1969). He experimented with isolated photosynthetic leaf cells and other functionally differented cells and was unsuccessful, but nevertheless he predicted that "one could successfully cultivate artificial embryos from vegetative cells." He thus clearly established the concept of totipotency, and further indicated that "the technique of cultivating isolated plant cells in nutrient solution permits the investigation of important problems from a new experimental approach." On the basis of that 1902 address and his pioneering experimentation before and later, Haberlandt is justifiably recognized as the father of plant tissue culture. Greater detail on the early pioneering events in plant tissue culture can be found in White (1963), Bhojwani and Razdan (1983), and Gautheret (1985).

THE EARLY YEARS

Using a different approach Kotte (1922), a student of Haberlandt, and Robbins (1922) succeeded in culturing isolated root tips. This approach, of using explants with meristematic cells, led to the successful and indefinite culture of tomato root tips by White (1934a). Further studies allowed for root culture on a completely defined medium. Such root cultures were used initially for viral studies and later as a major tool for physiological studies (Street, 1969). Success was also achieved with bud cultures by Loo (1945) and Ball (1946).

Embryo culture also had its beginning early in this century, when Hannig in 1904 successfully cultured cruciferous embryos and Brown in 1906 barley embryos (Monnier, 1995). This was followed by the successful rescue of embryos from nonviable seeds of a cross between *Linum perenne* × *L. austriacum* (Laibach, 1929). Tukey (1934) was able to allow for full embryo development in some early-ripening species of fruit trees, thus providing one of the earliest applications of *in vitro* culture. The phenomenon of precocious germination was also encountered (LaRue, 1936).

The first true plant tissue cultures were obtained by Gautheret (1934, 1935) from cambial tissue of *Acer pseudoplatanus*. He also obtained success with similar explants of *Ulmus campestre*, *Robinia pseudoacacia*, and *Salix capraea* using agar-solidified medium of Knop's solution, glucose, and cysteine hydrochloride. Later, the availability of indole acetic acid and the addition of B vitamins allowed for the more or less simultaneous demonstrations by Gautheret (1939) and Nobécourt (1939a) with carrot root tissues and White (1939a) with tumor tissue of a *Nicotiana glauca* × *N. langsdorffii* hybrid, which did not require auxin, that tissues could be continuously grown in culture and even made to differentiate roots and shoots (Nobécourt, 1939b; White, 1939b). However, all of the initial explants used by these pioneers included meristematic tissue. Nevertheless, these findings set the stage for the dramatic increase in the use of *in vitro* cultures in the subsequent decades.

The Era of Techniques Development

The 1940s, 1950s, and 1960s proved an exciting time for the development of new techniques and the improvement of those already available. The application of coconut water (often referred as coconut milk) by Van Overbeek *et al.* (1941) allowed for the culture of young embryos and other recalcitrant tissues, including monocots. As well, callus cultures of numerous species, including a variety of woody and herbaceous dicots and gymnosperms as well as crown gall tissues, were established (see Gautheret, 1985). Also at this time, it was recognized that cells in culture underwent a variety of changes, including loss of sensitivity to applied auxin or habituation (Gautheret, 1942, 1955) as well as variability of meristems formed from callus (Gautheret, 1955; Nobécourt, 1955). Nevertheless, it was during this period that most of the *in vitro* techniques used today were largely developed.

Studies by Skoog and his associates showed that the addition of adenine and high levels of phosphate allowed nonmeristematic pith tissues to be cultured and to produce

shoots and roots, but only in the presence of vascular tissue (Skoog & Tsui, 1948). Further studies using nucleic acids led to the discovery of the first cytokinin (kinetin) as the breakdown product of herring sperm DNA (Miller *et al.*, 1955). The availability of kinetin further increased the number of species that could be cultured indefinitely, but perhaps most importantly, led to the recognition that the exogenous balance of auxin and kinetin in the medium influenced the morphogenic fate of tobacco callus (Skoog & Miller, 1957). A relative high level of auxin to kinetin favored rooting, the reverse led to shoot formation, and intermediate levels to the proliferation of callus or wound parenchyma tissue. This morphogenic model has been shown to operate in numerous species (Evans *et al.*, 1981). Native cytokinins were subsequently discovered in several tissues, including coconut water (Letham, 1974). In addition to the formation of unipolar shoot buds and roots, the formation of bipolar somatic embryos (carrot) were first reported independently by Reinert (1958, 1959) and Steward *et al.* (1958).

The culture of single cells (and small cell clumps) was achieved by shaking callus cultures of *Tagetes erecta* and tobacco and subsequently placing them on filter paper resting on well-established callus, giving rise to the so-called nurse culture (Muir *et al.*, 1954, 1958). Later, single cells could be grown in medium in which tissues had already been grown, i.e., conditioned medium (Jones *et al.*, 1960). As well, Bergmann (1959) incorporated single cells in a 1-mm layer of solidified medium where some cell colonies were formed. This technique is widely used for cloning cells and in protoplast culture (Bhojwani & Razdan, 1983). Kohlenbach (1959) finally succeeded in the culture of mechanically isolated mature differentiated mesophyll cells of *Macleaya cordata* and later induced somatic embryos from callus (Kohlenbach, 1966). The first large-scale culture of plant cells was reported by Tulecke and Nickell (1959), who grew cell suspensions of *Ginkgo*, holly, *Lolium*, and rose in simple sparged 20-liter carboys. Utilizing coconut water as an additive to fresh medium, instead of using conditioned medium, Vasil and Hildebrandt (1965) finally