HAPLOIDY IN GENUS LOTUS (FABACEAE)

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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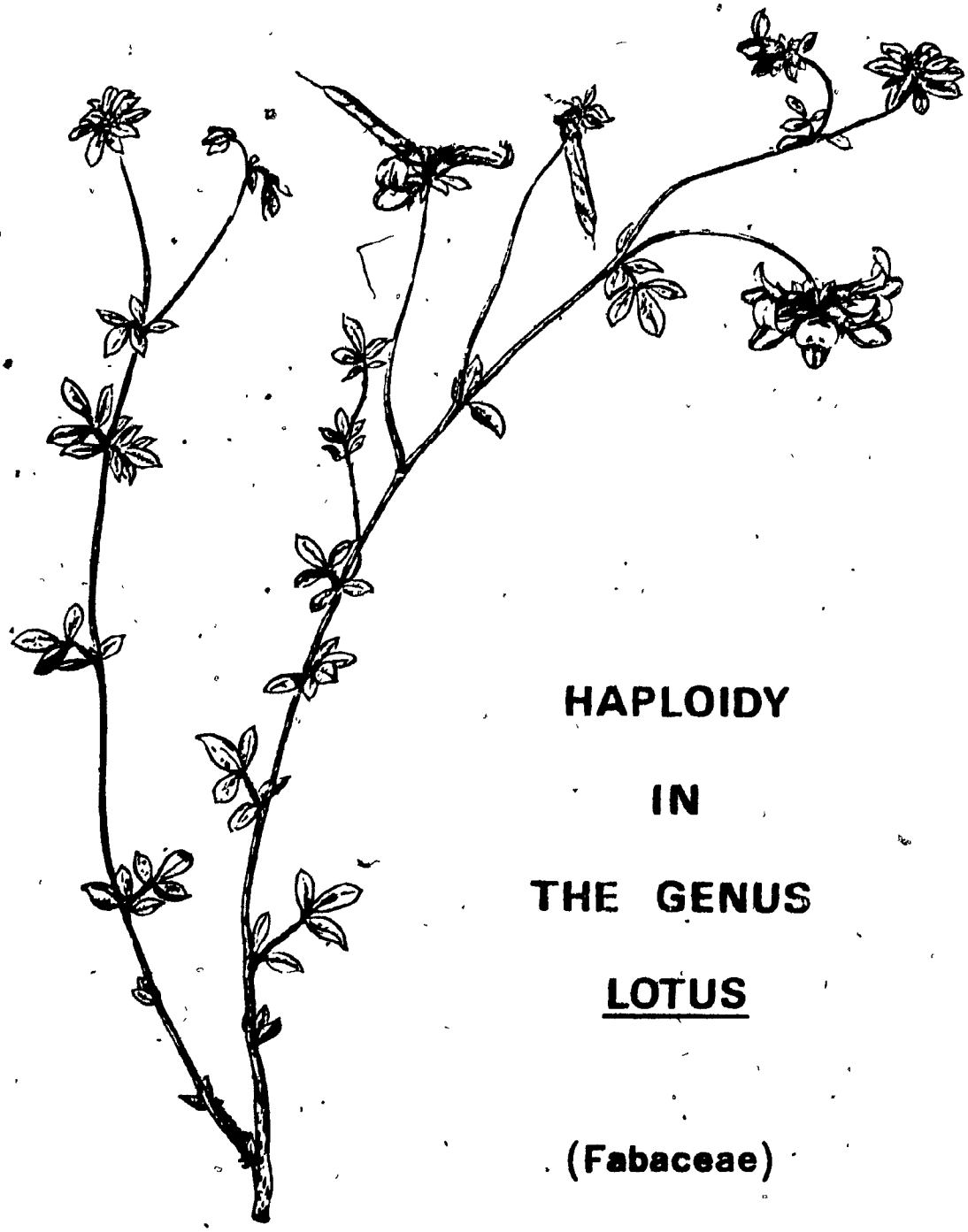
Montreal

August 1979



HAPLOIDY IN THE GENUS LOTUS

(Fabaceae)

DEDICATION

A mon père

J.-E. Lucien Séguin

(1911-1972)

"Une vie d'homme [et de femme] ne se justifie que par l'effort, même malheureux, vers le mieux comprendre. Et le mieux comprendre, c'est le mieux adhérer. Plus je comprends, plus j'aime, car tout ce qui est compris est bien."

Le matin des magiciens

J. Bergier et L. Pauwels

CLAIM TO ORIGINAL RESEARCH

The studies reported in this thesis constitute the original work of the author and represent her contribution to the subject of haploidy in the genus Lotus.

Reported for the first time are the following items:

1. The *in vitro* culture of four tetraploid Lotus species, namely, L. angustissimus L., L. emeroides Murray, L. mascaensis Buchard, and L. villosus Forsk.
2. The differentiation from anther culture of a mixoploid L. corniculatus plant which contained 2x, 4x, and 8x cells and the induction of multinucleate or multicellular pollen grains in cultured anthers of L. emeroides and L. villosus.
3. The *in vitro* culture of ovaries and ovules of eleven Lotus species and three hybrids, and the induction of morphogenesis (roots, shoots, and plantlets) from callus-derived cell lines.
4. The observation of cells with reduced ploidies in roots produced from callus tissue derived from a cultured ovary of L. emeroides.
5. The *in vitro* culture of inflorescences of four Lotus species and hybrids.
6. The cytological examination of twin seedlings of L. corniculatus cultivar Mirabel.

ACKNOWLEDGEMENTS

The author sincerely thanks her supervisor, Dr. W. F. Grant, Genetics Laboratory, Department of Plant Science, Macdonald College, McGill University, for his expert guidance throughout the course of these studies, and for his inexhaustible patience.

The author also wishes to acknowledge Ms. Siu-Wah Chung and Ms. Diane L. Benoit for their unfailing friendship and moral support, and my colleagues, Ms. Alina E. Russell and Mr. Don K. Zura, for their precious contributions to the preparation of this thesis, and also for bearing with the author.

The technical assistance of Ms. Marie Gourley is greatly appreciated. The author also acknowledges the devoted efforts of Ms. Rosemary J. Haraldsson and Ms. Lise Séguin-Lala towards the preparation and the typing of the manuscript.

The financial assistance of the National Sciences and Engineering Research Council of Canada to Dr. Grant, and of the Direction Générale de l'Enseignement Supérieur, Ministère de l'Éducation, Gouvernement du Québec, to the author, is gratefully acknowledged.

ABSTRACT

Ph.D. GINETTE SEGUIN Biology

HAPLOIDY IN THE GENUS LOTUS (FABACEAE)

Attempts were made to recover haploid plants from sixteen Lotus species and hybrids by means of in vitro culture techniques and twin screening. Multinucleate or multicellular pollen grains were observed in cultured anthers of L. emeroides Murray and L. villosus Forsk. Cytological examination of a L. corniculatus L. (2n=24) plant regenerated from anther culture revealed a mixoploid condition including haploid (dihaploid) 2x cells, as well as 4x and 8x cells. A callus cell line derived from a cultured ovary of L. emeroides (2n=28) produced roots which contained 2x cells and aneuploid cells with somatic chromosome numbers less than 28. Morphogenesis (roots, shoots, or plantlets) was induced from both anther and ovary callus cell lines. Haploids were not induced from microspore, pollen tube, or inflorescence culture. Screening for polyembryonic seeds led to the recovery of twin seedlings of L. pedunculatus var. villosus Lamotte and L. corniculatus cv. Mirabel. The twins examined were all tetraploid.

SOMMAIRE

Ph.D. GINETTE SEGUIN Biologie

HAPLOIDIE CHEZ LE GENRE *LOTUS* (FABACEAE)

Les études entreprises visaient à induire, chez seize espèces et hybrides appartenant au genre *Lotus*, la formation de plantes haploïdes par l'application de techniques culturales *in vitro*, ainsi qu'à découvrir des haploïdes chez des graines polyembryonées. Des grains de pollen plurinucléés et pluricellulaires furent observés dans des cultures d'anthères de *L. emeroides* Murray et de *L. villosus* Forsk. L'examen cytologique d'une plante de *L. corniculatus* L. ($2n=24$) regénérée d'une culture d'anthères a révélé une constitution mixoploïde, comportant des cellules haploïdes ($2x$), tétraploïdes ($4x$) et octoploïdes ($8x$). Une lignée cellulaire dérivée d'un cal d'ovaire de *L. emeroides* ($2n=28$) a produit des racines dont l'examen cytologique a démontré la présence de cellules haploïdes ($2x$), ainsi que de cellules aneuploïdes dont les nombres chromosomiques s'échelonnaient de 14 à 28. La formation de racines, de tiges et de plantules à partir de lignées cellulaires dérivant de cals d'anthères et d'ovaires fut également observée. Les cultures de pollen isolé de l'anthère, de tubes polliniques et d'inflorescences n'ont pas produit de plantes haploïdes. La recherche de graines polyembryonées a permis d'obtenir des plantules jumelles chez les deux espèces suivantes, *L. pedunculatus* var. *villosus* Lamotte et *L. corniculatus* cv. Mirabel. Toutes les plantules issues de graines polyembryonées étaient tétraploïdes.

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LIST OF ABBREVIATIONS

AS	Adenine sulfate
B5	Gamborg, Miller, and Ojima's culture medium (1968)
BA	Benzyladenine
BAP	Benzylaminopurine
BN	Benazolin
CM	Coconut milk
2,4-D	2,4-Dichlorophenoxyacetic acid
α,β-D	α,β-Dichloropropionic acid
GA	Gibberellic acid
GM	Guha and Maheshwari's culture medium (1966)
IAA	Indoleacetic acid
K	Kinetin (6-furfurylaminopurine)
KC	Kasperbauer and Collins' culture medium (1972)
LS	Lismaier and Skoog's culture medium (1965)
LST	Lin and Staba's culture medium (1961)
MS	Murashige and Skoog's culture medium (1962)
NAA	α-Naphthaleneacetic acid
NAA	Naphthylacetic acid
VM	Veliky and Martin's culture medium (1970)
Z	Zeatin

INTRODUCTION

Since the discovery by Blakeskee et al. (1922) more than half a century ago of the first haploid plant in the species Datura stramonium L., considerable research has been devoted to the study of haploidy. The fact that haploid plants may constitute invaluable tools in various research areas such as cytology, cytogenetics, plant physiology, genetics, and more particularly in the fields of plant breeding and mutation research, has been emphasized by several investigators (Melchers and Labib, 1970; Melchers, 1972; Vasil and Nitsch, 1975; Nitzsche and Wenzel, 1977).

Haploids have proven of great value in several instances, as illustrated by the work of Riley and Chapman (1958) where the study of meiotic pairing in polyhaploids and aneupolyhaploids of hexaploid wheat lead to the characterization of a genetic system controlling homoeologous pairing. Haploids have also been useful in the acceleration of breeding programs; spontaneous or induced haploid plants may be doubled by means of various techniques to produce isogenic lines which can readily be incorporated into breeding schemes, as for instance, in maize (Chase, 1952), asparagus (Thévenin, 1974), rice (Anonymous, 1978k), and rape (Stringham, cited in Keller and Stringham, 1978).

Although spontaneous haploid plants have been recorded in a large number of species (Ivanov, 1938; Kostoff, 1942; Kehr, 1951; Chase, 1952; Kimber and Riley, 1963; Magoon and Khanna, 1963), their relatively low incidence has impelled scientists to investigate artificial means of induction.

Irradiation of either the embryo sac (Gerassimova, 1936;

Ehrensberger, 1948) or the pollen used for pollinating normal plants (for a review of the literature, see Lacadena, 1974), wounding and traumatic injury (Davis, 1931; Ivanov, 1938; Chase, 1969), and various temperature treatments (Blakeslee et al., 1922; Müntzing, 1937; Nordenskiöld, 1939; Winton and Einspahr, 1968) have proven more or less effective in inducing parthenogenesis.

Chemical agents, including those which inactivate the sperm nuclei without preventing the growth of the pollen tube and the stimulation of division of the egg cell, for instance toluidine blue (Al-Yasiri and Rogers, 1971; Illies, 1974), and those which have been reported to cause somatic reduction, such as colchicine (Sanders and Franzke, 1962; Simantel and Ross, 1964), have been of limited value in producing haploid plants.

A more effective method for obtaining parthenogenetic individuals has consisted of performing interspecific crosses; in this regard, remarkable success has been achieved with Solanum species (for a review of the literature on interspecific hybridization, see Kimber and Riley, 1963; Magoon and Khanna, 1963; Chase, 1969; Rowe, 1974).

Studies involving somatic elimination of chromosomes (Kasha, 1974) and semigamy (Turcotte and Feaster, 1974) have also produced successful results.

And finally, the production of haploid plants has also been obtained via in vitro techniques which include anther, pollen, and ovary culture, as well as others.

Haploids have never been reported in the genus Lotus and it has been the aim of this study to investigate means of recovering spontaneous and induced haploid plants in this genus.

I

LITERATURE REVIEW

Spontaneous haploid plants arising from polyembryonic seeds and in vitro-induced haploids have been reported by a number of investigators. In this review, only the relevant literature pertaining to these two aspects of haploidy will be considered.

A. Anther and Pollen Culture

Since Guha and Maheshwari's report (1966) confirming that the embryo-like structures observed in cultured anthers of Datura innoxia Mill. (Guha and Maheshwari, 1964) had originated from microspores, the in vitro culture of anthers or isolated microspores as a means of producing haploid plants has aroused considerable interest.

In vitro culture of anthers was not a novel technique, as several investigators had cultured anthers previously in order to study the various stages of pollen development (Shimakura, 1934; Gregory, 1940; St.Gimesi et al., 1949; Taylor, 1949, 1950; Lima-de-Faria, 1950; Sparrow et al., 1955; Vasil, 1957, 1959; Walker, 1957; Vasil, 1963). However, these studies did not lead to the recovery of haploid callus and plantlets.

LaRue (cited in Tulecke, 1959) cultured mature pollen grains of 33 angiosperm genera with the view of obtaining a callus tissue, but none of the experiments proved successful.

Several reviews have recently been devoted to anther and pollen culture of angiospermous and gymnospermous species (Acharya and Ramji, 1977; Collins, 1977; Niizeki, 1977; Nitzsche and Wenzel, 1977; Reinert and

Bajaj, 1977; Sink and Padmanabhan, 1977; Collins, 1979). Various levels of success have been attained with the numerous species assayed. So far, in angiosperms, 91 species and hybrids representative of 37 genera in 15 families have successfully produced androgenetic haploids (Tables I, II).

The physiological basis underlying the embryogenic behavior of the cultured microspores remains obscure and, in recent years, there has been a great deal of research devoted to the elucidation of the factors involved in embryoid induction.

The importance of these studies is crucial, as their outcome may bring an understanding of the reasons why certain families, the Solanaceae for instance, can be easily induced, whereas others, such as the Fabaceae, offer tremendous resistance.

A detailed account of the various attempts to induce haploidy by means of anther and pollen cultures is given in the following pages.

Nota

The author has, as far as possible, attempted to use the correct latin names; invalid binomials, as published in the literature, have been placed between parentheses.

TABLE I. Monocotyledonous species from which haploid plantlets and plants have been recovered through anther and pollen culture

Family	Genus	Species	References
LILIACEAE			
	Asparagus		
		A. officinalis L.	Pelletier et al., 1972; Doré, 1974; Thévenin, 1974.
	Lilium		
		L. longiflorum Thunb.	Sharp et al., 1972.
POACEAE			
	Aegilops		
		A. caudata L. X A. umbellata Zhuk.	Kimata and Sakamoto, 1972
	Bromus		
		B. inermis Leyss.	Saito et al., 1973; Zenkteler, 1976.
	Festuca		
		F. elatior L. var. arundinacea (Schreb.) Wimmer (F. arundinacea Schreb.)	Kasperbauer and Buckner, 1979.
	Festuca X Lolium		
		F. elatior L. (2x) X L. multiflorum Lam. (2x)	Nitzsche, cited in Nitzsche and Wenzel, 1977.
		F. elatior L. (4x) X L. multiflorum Lam. (4x)	Nitzsche, cited in Nitzsche and Wenzel, 1977.

Continued...

TABLE I. (Continued)

Family	Genus	Species	References
	Hordeum		
		H. vulgare L.	Clapham, 1973; Malepszy and Grunewaldt, 1974; Grunewaldt and Malepszy, 1975; Foroughi-Wehr et al., 1976; Gaul et al., 1976a; Friedt et al., 1977 (4x); Wilson, 1977; Mix et al.,1978; Wilson et al., 1978a.
	Lolium		
		L. multiflorum Lam.	Clapham, 1971; Niizeki, 1977; Nitzsche, cited in Nitzsche and Wenzel, 1977.
	Lolium X Festuca		
		L. multiflorum Lam (4x) X F. elatior L. var. arundinacea (Schreb.) (F. arundinacea Schreb.)	Nitzsche, 1970.
	Oryza		
		O. sativa L.	Niizeki, 1968; Niizeki and Oono, 1968; Harn, 1969; Nishi and Mitsuoka, 1969; Guha et al., 1970; Iyer and Raina, 1972; Woo and Tung, 1972; Guha-Mukjerjee, 1972; Fouletier, 1974; Chaleff et al., 1975; Oono, 1975; Woo and Su, 1975; Chen and Lin, 1976; Yin et al., 1976; Chen, 1977.
	Secale		
		S. cereale L.	Thomas et al., 1975; Wenzel et al, 1976a; Orlikowska, 1977; Wenzel et al., 1977.

Continued...

TABLE I. (Continued)

Family	Genus	Species	References
	Setaria		
		S. italica Beauv.	- Ban et al., 1971
	X Triticosecale Wittmack		Wang et al.,1973b; Sun et al., 1974; Bernard et al., 1976; Ono and Larter, 1976; Bernard, 1977; Orlikowska, 1977; Dorosiev et al., 1978.
	Triticum		
		T. aestivum L.	Ouang et al., 1973; Picard and de Buyser, 1973, 1975, 1977; Wang et al., 1973a; Craig, 1974; Shimada and Makino, 1975; Heszky and Mesch, 1976; Chuang et al.,1978; Han et al., 1978.
		T. dicoccoides Koerm.	Chu, 1978.
	Triticum X Agropyron		
		T. aestivum L. (T. vulgare Vill.) X A. glaucum Roem. & Schult.	Wang et al., 1975b.
	Zea		
		Z. mays L.	Anonymous, 1978e,f; Ku et al.,1978a,b; Li and Ts'ui, 1978; Miao et al., 1978.

TABLE II. Dicotyledonous species from which haploid plantlets and plants have been recovered through anther and pollen culture

Family	Genus	Species	References
APOCYNACEAE			
	Vinca		
		V. rosea L. (Catharanthus roseus Don.)	Abu-Mandoor and Czygan, cited in Nitzsche and Wenzel, 1977.
ASTERACEAE			
	Gerbera		
		G. jamesonii Bolus	Preil et al., 1978.
BRASSICACEAE			
	Arabidopsis		
		A. thaliana (L.) Heynh.	Gresshoff and Doy, 1972a; Avetisov, 1976.
	Brassica		
		B. campestris L.	Anonymous, 1975.
		B. chinensis L.	Chung et al., 1978.
		B. napus L.	Thomas and Wenzel, 1975b; Wenzel et al., 1977b; Keller and Armstrong, 1978.
		B. oleracea L.	Kameya and Hinata, 1970; Chung et al., 1978.
		B. pekinensis Rupr.	Teng and Kuo, 1978.
		B. oleracea L. X B. alboglabra	Kameya and Hinata, 1970.

Continued...

TABLE II. (Continued)

Family	Genus	Species	References
CHENOPODIACEAE			
	Beta		
		B. vulgaris L.	Pao et al., 1978.
EUPHORBIACEAE			
	Hevea		
		H. brasiliensis Muell. Arg.	Chen et al., 1978a,b; Anonymous, 1978c.
GERANIACEAE			
	Pelargonium		
		P. hortorum Bailey	Abo El-Nil and Hildebrandt, 1973.
GESNERIACEAE			
	Saintpaulia		
		S. ionantha Wendl.	Hughes et al., 1975.
HIPPOCASTANACEAE			
	Aesculus		
		A. hippocastanum L.	Radojević, 1978.
PAPAVERACEAE			
	Papaver		
		P. bracteatum Lindl.	Abu-Mandoor and Czygan, cited in Nitzsche and Wenzel, 1977; Corduan, cited in Nitzsche and Wenzel, 1977.

Continued...

TABLE II. (Continued)

Family	Genus	Species	References
SALICACEAE			
	Populus		
		P. berolinensis C. Koch	Lu (C.-H.) et al., 1978.
		P. nigra L.	Wang et al., 1975a.
		P. pekinensis L. Henry	Lu (C.-H.) et al., 1978.
		P. pseudosimonii	Lu (C.-H.) et al., 1978.
		P. simonii Carr.	Lu (C.-H.) et al., 1978.
		P. berolinensis C. Koch X P. nigra L. var. pyramidalis Spach. (P. pyramidalis Salisb.)	Lu (C.-H.) et al., 1978.
		P. canadensis Moench X P. koreana Rehder	Lu (C.-H.) et al., 1978.
		P. harbinenais X P. nigra L. var. pyramidalis Spach. (P. pyramidalis Salisb.)	Lu (C.-H.) et al., 1978.
		P. pseudosimonii X P. nigra L. var. pyramidalis Spach. (P. pyramidalis Salisb.)	Lu (C.-H.) et al., 1978.
		P. simonii Carr. X P. nigra L.	Lu (C.-H.) et al., 1978.
		P. simonii Carr. X P. nigra L. var. pyramidalis Spach. (P. pyramidalis Salisb.)	Lu (C.-H.) et al., 1978.

Continued...

TABLE II. (Continued)

Family	Genus	Species	References
SCROPHULARIACEAE			
	Digitalis		
		D. lanata Ehrh.	Corduan, cited in Nitzsche and Wenzel, 1977.
		D. purpurea L.	Corduan and Spix, 1975.
SOLANACEAE			
	Atropa		
		A. belladonna L.	Zenkteler, 1971; Narayanaswamy and George, 1972; Misiura and Zenkteler, 1973; Rashid and Street, 1973.
	Capsicum		
		C. frutescens L. (C. annuum L.)	George and Narayanaswamy, 1973; Kuo et al., 1973; Wang et al., 1973b; Wang et al., 1978a,b.
	Datura		
		D. innoxia Mill.	Guha and Maheshwari, 1966, 1967; Nitsch and Nitsch, 1970; Engvild et al., 1972; Nitsch, 1972; Sopory and Maheshwari, 1972; Geier and Kohlenback, 1973; Nitsch and Norreel, 1973; Collins et al., 1974a; Nitsch, 1974b; Sunderland et al., 1974; Sunderland and Roberts, 1976; Sangwan-Norreel, 1977.

Continued...

TABLE II. (Continued)

Family	Genus	Species	References
		D. metel L.	Chandy and Narayanaswamy, 1971; Narayanaswamy and Chandy, 1971; Iyer and Raina, 1972; Nitsch, 1972.
		D. meteloides Dunal	Kohlenbach and Geier, 1972; Nitsch, 1972; Geier and Kohlenbach, 1973.
		D. muricata Link	Nitsch, 1972.
		D. wrightii Regel	Kohlenbach and Geier, 1972.
	Hyoscyamus		
		H. albus L.	Raghavan, 1975.
		H. niger L.	Corduan, 1975; Raghavan, 1975.
		H. pusillus L.	Raghavan, 1975.
	Lycium		
		L. halimifolium Mill.	Zenkteler, 1972.
	Lycopersicon		
		L. esculentum L.	Gresshoff and Doy, 1972b.
	Nicotiana		
		N. alata Link & Otto (N. affinis Hort.)	Nitsch, 1969, 1972; Nitsch and Nitsch, 1969, 1970.
		N. attenuata Torr.	Collins and Sunderland, 1973, 1974.
		N. clevelandii A. gray	Vyskot and Novák, 1974.
		N. glutinosa L.	Nitsch, 1969 (4x); Nakamura and Itagaki, 1973; Niizeki, 1975; Tomes and Collins, 1976.

Continued...

TABLE II. (Continued)

Family	Genus	Species	References
		N. knightiana Goodspeed	Collins and Sunderland, 1973, 1974.
		N. otophora Gris.	Collins et al., 1972; Nakamura and Itagaki, 1973; Vagera, 1978.
		N. paniculata L.	Bourgin and Missonier, 1973; Tomes and Collins, 1976; Vagera, 1978.
		N. raimondii Macb.	Collins and Sunderland, 1973, 1974.
		N. rustica L.	Nitsch, 1969, 1972; Nitsch and Nitsch, 1969; Nakamura and Itagaki, 1973; Tomes and Collins, 1976.
		N. sylvestris Spegazzini & Comes	Bourgin and Nitsch, 1967; Nitsch et al.,1968; Nitsch, 1969, 1972; Nitsch and Nitsch, 1969; Niizeki, 1975; Tomes and Collins, 1976; Vagera, 1978.
		N. tabacum L.	Bourgin and Nitsch, 1967; Nakata and Tanaka, 1968; Nitsch et al.,1968; Nitsch, 1969, 1972; Nitsch and Nitsch, 1969; Sunderland and Wicks, 1969; Nilsson-Tillgren and Wettstein-Knowles, 1970; Devreux et al., 1971; Sunderland and Wicks, 1971; Burk et al., 1972; Collins et al., 1972; Kasperbauer and Collins, 1972; Corduan, 1973; Dunwell and Perry, 1973; Anagnostakis, 1974; Engvild, 1974; Kasperbauer and Collins, 1974; Mattingly and Collins, 1974; Niizeki, 1974; Nitsch, 1974a; Tsikov et al., 1974;

Continued...

TABLE II. (Continued)

Family	Genus	Species	References
			Zagorska et al., 1974, 1978; Gorenflot et al., 1975; Niizeki, 1975; Niizeki and Kita, 1975 a,b; Novák and Vyskot, 1975; Reinert et al.,1975; Zagorska, 1975; Duncan and Heberle, 1976; Sarvella et al., 1976; Tomes and Collins, 1976; Niizeki, 1977; Sunderland and Roberts, 1977b; Vagera, 1978.
		N. velutina	Vagera, 1978.
		N. forgetiana Sander X N. alata Link & Otto (N. sanderae Hort.)	Vyskot and Novák, 1974; Vagera, 1978.
		N. sylvestris Spegazzini & Comes X N. tomentosiformis Goodspeed	Vagera, 1978.
	Petunia		
		P. hybrida Vilm	Maizonnier, 1973; Raquin, 1973; Wagner and Hess, 1974; Sangwan and Norreel, 1975b.
		P. axillaris BSP X P. hybrida Vilm.	Raquin and Pilet, 1972.
	Scopolia		
		S. physaloides Dun.	Wernicke and Kohlenbach, 1975.
	Solanum		
		S. bulbocastanum Dun.	Irikura, 1975.
		S. demissum Lindl.	Irikura, 1975.

Continued...

TABLE II. (Continued)

Family	Genus	Species	References
		S. dulcamara L.	Zenkteler, 1973.
		S. fendleri Heurck. & Muell.	Irikura, 1975.
		S. hjertingii J.G. Hawkes	Irikura, 1975.
		S. melongena L.	Anonymous, 1978d,j; Isouard et al., 1979.
		S. nigrum L.	Harn, 1971, 1972a,b; Irikura, 1975.
		S. phureja Juz. & Buk.	Irikura, 1975.
		S. polytrichon Rydb.	Irikura, 1975.
		S. stenotomum Juz. & Buk.	Irikura, 1975.
		S. stoloniferum Schlecht. & Bouché	Irikura, 1975.
		S. tuberosum L.	Dunwell and Sunderland, 1973; Irikura, 1975; Foroughi-Wehr et al., 1977; Sopory et al., 1978.
		S. verrucosum Schlecht.	Irikura and Sakaguchi, 1972; Irikura, 1975.
		S. verrucosum Schlecht. X S. chacoense Bitt.	Irikura, 1975.
		S. verrucosum Schlecht. X S. tuberosum L.	Irikura, 1975.
THEACEAE			
	Thea		
		T. sinensis L. (Camellia thea Link)	Okano and Fuchinoue, 1970.

Gymnospermae

There are very few reports of pollen culture studies performed on gymnospermous species. Most studies have been concerned with the culture of mature pollen grains and the growth of callus tissue, usually haploid. Organ differentiation has not been described.

Cupressaceae

Cupressus arizonica Greene

Callus tissue was obtained from cultured microsporophylls of the rough-barked Arizona cypress, C. arizonica (Duhoux and Norreel, 1974). The authors believed that the tissue originated from the microspores and that the haploid and the diploid cells found in this callus had a common origin.

Juniperus species

Duhoux and Norreel (1974) reported that callus tissues, presumably of pollen origin, developed from microsporophylls both of the Chinese juniper, J. chinensis L., and the common juniper, J. communis L. The pollen was cultured on several different media. Haploid as well as diploid cells were observed in these calluses.

Thuja orientalis L.

The formation of a callus tissue from in vitro-cultured pollen grains of oriental arbor-vitae was described by Rao and Mehta (1969); the authors, however, did not determine the ploidy of the cells.

Ephedraceae

Ephedra foliata Boiss.

Konar (1963) reported the development of haploid callus tissue from in vitro-cultured mature pollen of E. foliata. Two patterns of development were noted; the pollen grains either elongated to form multicellular filaments or enlarged to produce discoid multicellular masses. The author hypothesized that the callus tissue originated from the generative cell.

Ginkgoaceae

Ginkgo biloba L.

A decade before Guha and Maheshwari's discovery, Tulecke (1953, 1957) was able to derive a haploid tissue from in vitro culture of mature pollen of G. biloba. The author concluded from his studies that the callus had originated, for the major part, from divisions of the pollen tubes. Cytological examination of the tissue showed cells with haploid (n=12), diploid, tetraploid, and heteroploid numbers of chromosomes.

Pinaceae

Larix species

Haploid calluses were recovered from cultured microsporophylls of two Larix species, L. laricina Koch, the American larch, and L. decidua Mill., the European larch (Bonga, 1976).

Pinus species

Bonga and Fowler (1970) and Bonga (1974) attempted the in vitro

culture of microsporophylls of red pine, P. resinosa Ait., using Brown and Lawrence's medium (1968). Undifferentiated haploid callus developed from the young microspores, but callus formation could not be initiated from mature pollen grains. Centrifugation of the male strobili prior to culture stimulated callus development (Bonga and McInnis, 1975). Haploid calluses were also recovered from microsporophyll cultures of the Swiss mountain pine, P. mugo Turra, and the Austrian pine, P. nigra Arnold (Bonga, 1976).

Taxaceae

Taxus sp.

The growth of callus tissue from cultured mature pollen grains of a Taxus species was successfully achieved by LaRue (cited in Tulecke, 1959). However, the ploidy of the cells was not reported.

Torreya nucifera Sieb. and Zucc.

Tulecke and Sehgal (1963) reported the development of a tissue from cultured pollen grains of T. nucifera. Division of the tube cells by a process of budding was observed, as well as multinucleate and septate tube cells.

Angiospermae

Anther and pollen culture has been attempted in several angiospermous species; a variety of results have been obtained, ranging from haploid plant recovery to complete failure of pollen induction. A detailed review of the literature follows.

Monocotyledoneae

Amaryllidaceae

Clivia nobilis L.

Debergh (1976) reported the abnormal behavior of microspores in cultured anthers of C. nobilis. Multinucleate pollen grains were observed, sometimes with differently sized, shaped, or stained nuclei. The presence of multicellular grains was not reported.

Commelineceae

Tradescantia paludosa E. Anders & R. E. Woodson

Pollen divisions have been observed in the cultured anthers of one species, T. paludosa (Kaufman, 1972). No plantlets were regenerated from the cultures.

Iridaceae

Freesia sp.

Pollen grains with four to five cells were observed in anthers of a yellow-flowered cultivar of Freesia cultured at either the uninucleate stage or just after the first pollen mitosis on GM medium supplemented with a vitamin complex, 1 ppm NAA, and 10% CM (Sunderland

et al., 1971). However, these multicellular pollen grains did not develop further.

Liliaceae

Allium ursinum L.

Anther culture of A. ursinum was attempted by Velican (1978) with little success. Callus developed from a single anther out of the 400 cultured. Further investigation could not be done because of contamination. Examination of the non-callused anthers showed that embryogenesis was an extremely rare event.

Asparagus officinalis L.

The successful regeneration of haploid asparagus plantlets was first described by Pelletier et al. (1972). The authors reported that the best results were obtained with anthers cultured at the uninucleate stage on a synthetic medium supplemented with NAA, 2,4-D, and BAP. The growth regulator 2,4-D was found essential to the induction of pollen mitoses but was not required for further stages of development. However, the regenerated plantlets could not be grown into mature plants. Hondelmann and Wilberg (1973) were unable to duplicate these findings.

A report of mature haploid asparagus plants obtained via anther culture was later made by Doré (1974). Diploidized androgenic plants of asparagus have been already introduced into breeding programs (Thévenin, 1974).

Lilium longiflorum Thunb.

Sharp et al. (1972) described the induction of haploid plantlets of white trumpet lily. Haploid callus tissue, which was produced from

anthers cultured on a modified MS medium, differentiated bulblets which subsequently developed into plantlets.

Tulipa gesneriana L.

Unsuccessful attempts to induce *in vitro* androgenesis in tulip anthers were reported by Haccius (1972).

Palmaceae

Cocos nucifera L.

Sunderland *et al.* (1971) reported that, in cultured anthers of the coconut tree, the microspores degenerated very rapidly and failed to enter the first pollen mitosis. Unsuccessful results were also briefly mentioned by Iyer and Raina (1972).

Poaceae

Aegilops species

Kimata and Sakamoto (1971, 1972) reported the regeneration of albino plantlets from anther calluses of a synthetic amphiploid, *A. caudata* L. X *A. umbellulata* Zhuk. The anthers were cultured on Miller's medium (1963) supplemented with 2.21 ppm 2,4-D. Chromosome counts performed on the root tips of these plants showed that they possessed the haploid chromosome number of 14. The development of callus tissue from cultured pollen grains of *A. variabilis* Eig was also observed.

The authors were unable to induce callus formation from anthers of nine *Aegilops* species, namely, *A. caudata* L., *A. comosa* Sibth. et Sm., *A. crassa* Boiss., *A. cylindrica* Host., *A. ovata* L., *A. speltoides* Tausch, *A. squarrosa* L., *A. triuncialis* L., and *A. umbellulata* Zhuk.

Agropyron species

The formation of callus from cultured pollen grains of two Agropyron species, A. elongatum Host. ex Beauv. and A. intermedium Beauv., was reported by Kimata and Sakamoto (1971). Calluses were not induced from six other species cultured, namely, A. campestre Godr. and Gren., A. junceum Beauv., A. littorale Dum., A. repens (L.) Beauv., A. smithii Rydb., and A. trichophorum C. Richt.

Multicellular microspores were observed by Zenkteler and Misiura (1974a) and Zenkteler et al. (1975) in anthers of quack grass, A. repens, cultured on MS and LS media supplemented with various growth regulators and sucrose concentrations.

Anthoxanthum odoratum L.

Anther culture of sweet vernal-grass was attempted by Zenkteler and Misiura (1974a) without success. The authors observed only degenerating microspores in the cultured material.

Arrhenatherum pratense

Zenkteler and Misiura (1974a) were unable to induce pollen divisions in cultured anthers of this species.

Avena sativa L.

Keller (1974) cultured a few anthers of oats, cv. Garry, without any success. After eight weeks, neither callus nor embryoids were observed.

Callus tissue was obtained from seven out of 12,000 anthers cultured on various media (Rines, 1978). The ploidy of the callus cells was not determined and morphogenesis was not reported.

S.-W. Chung (Macdonald College, Ste-Anne de Bellevue, Quebec; pers.

comm.) was able to induce the formation of calluses from three oat cultivars, namely, Cayuse, Stout, and Manod; she also observed multicellular development in the microspores.

Avenastrum pratense (L.) Opiz

Degenerating microspores were observed in cultured anthers of this species (Zenkteler and Misiura, 1974a).

Bromus species

Saito et al. (1973) successfully induced the formation of haploid plants of smooth brome-grass, B. inermis Leyss., from anther-derived callus tissues.

Pollen embryos (Zenkteler and Misiura, 1974a; Zenkteler et al., 1975) and haploid albino plants (Zenkteler, 1976) were recovered from anther cultures of B. inermis. The plants were produced from calluses which developed from anthers sown at the uninucleate stage on a basal MS medium, supplemented with 1 ppm K, 0.5 ppm IAA, 2 ppm 2,4-D, and 6% sucrose.

Niizeki (1977) was unable to induce callus formation on cultured anthers of B. inermis.

Zenkteler and Misiura (1974a) observed the presence of multinucleate microspores in cultured anthers of B. sterilis L.

Pollen degeneration in cultured anthers of B. catharticus Vahl (B. unioloides HBK) was also reported by the authors.

Dactylis glomerata L.

Niizeki (1977) failed to initiate callus formation from cultured anthers of orchard-grass, D. glomerata.

Festuca species

Only degenerating microspores were observed in cultured anthers of F. elatior L. var. arundinacea (Schreb.) Wimmer (F. arundinacea Schreb.), F. ovina L. var. glauca Koch (F. glauca Lam.), and F. longifolia Thuill. (Zenkteler and Misiura, 1974a).

A single callusing anther of F. arundinacea was recovered by Niizeki (1977) out of 806 anthers cultured on a variety of media. The callus, which was extremely slow-growing, finally degenerated after two months of incubation.

The recovery of haploid plants from anthers of F. arundinacea was recently reported by Kasperbauer and Buckner (1979). The donor plants were subjected to a cold treatment before the anthers or the entire spikelets were sown on a modified MS medium supplemented with 2,4-D. Haploids developed only from anthers that had not been removed from the spikelets.

Multicellular microspores were observed in cultured anthers of F. elatior L. var. elatior (F. pratensis Huds.) by Zenkteler and Misiura (1974a,b) and Zenkteler et al. (1975). Niizeki (1977) was unable to induce the development of callus tissue from anthers of this variety.

Festuca elatior L. X Lolium multiflorum Lam.

Nitzsche (cited in Nitzsche and Wenzel, 1977) reported the recovery of four albino and six green haploid plants from anther cultures of hybrids between F. elatior and L. multiflorum.

Hordeum species

Pollen callus from cultured anthers of barley, H. vulgare L., was first obtained by Clapham (1971); he later recovered androgenic

haploids (Clapham, 1973).

Multicellular pollen grains and pollen embryoids were described by Zenkteler and Misiura (1974a,b) and Zenkteler et al. (1975).

The recovery of haploid androgenetic plantlets was also reported by several investigators (see Table I).

Various aspects of barley anther culture have been investigated; these include studies on the determination of the most inducible pollen developmental stage (Gaul et al., 1976b), the stimulation of the frequency of pollen callus induction by the culture of whole spikes in liquid medium (Wilson, 1977; Wilson et al., 1978b), and the cytological observation of pollen embryogenesis (Bouharmont, 1977; Sun, 1978).

Anther culture of two other Hordeum species, H. bulbosum L. and H. jubatum L., was attempted by Zenkteler and Misiura (1974a). Multicellular pollen grains were observed in cultured anthers of H. bulbosum, whereas, only degenerating microspores were noted in H. jubatum cultures.

Lolium species

Clapham (1971) recovered haploid albino plantlets from anthers of Italian rye-grass, L. multiflorum Lam., cultured just prior to the first pollen mitosis on LS basal medium supplemented with 1 ppm NAA, 15% CM and 12% sucrose. Up to 20% of the anthers developed macroscopic calluses from which a small number of plantlets differentiated. A green haploid L. multiflorum plant was obtained by Nitzsche (cited in Nitzsche and Wenzel, 1977) from cultured anthers of this species. Niizeki (1977) also reported the recovery of a haploid L. multiflorum plant in which the pollen fertility (92.2%) was greater than the donor

plant (70.5%).

Anther-derived calluses of perennial rye-grass, L. perenne L., were studied by Niizeki (1977). He considered the calluses as having originated from the microspores. Multicellular pollen grains were also observed by Zenkteler and Misiura (1974a) in cultured anthers of L. perenne.

Anther culture of L. hybridum Opiz did not lead to callus or embryoid formation (Niizeki, 1977).

Lolium multiflorum Lam. X Festuca elatior L. var. arundinacea (Schreb.) Wimmer (F. arundinacea Schreb.)

Nitzsche (1970) reported the recovery of haploid plants from anther cultures of a hybrid between Italian rye-grass, L. multiflorum, and a dodecaploid meadow fescue.

Oryza sativa L.

The differentiation of haploid rice plantlets from anther-derived calluses was first reported by Niizeki (1968) and Niizeki and Oono (1968). The successful induction of androgenic haploids in different rice cultivars via a callus phase has since been described by several investigators (see Table I); the recovery of haploid plantlets obtained by direct embryogenesis has also been reported (Guha-Mukherjee, 1973).

Anther culture in rice is being extensively studied in China; for instance, considerable research is being directed towards the improvement of culture media and cultural conditions, the solution of the problematic origin of albino plantlets, and the incorporation of progenies of doubled haploids into breeding programs [for references, see Proc. Symp. on Anther Culture, Canton (1978a), more than 50 communications

on rice anther culture; also Proc. Symp. on Plant Tissue Culture, Peking (1978b), 16 papers].

Pennisetum glaucum R. Br. (P. typhoides (Burm.) Stapf & Hubb.)

Anther culture of pearl millet was attempted by Sree Ramulu et al. (1976a) without success. The authors tried a variety of culture media supplemented with various addenda. Neither the development of the microspores nor callus or embryoid formation were observed.

Nitsch (cited in Nitzsche and Wenzel, 1977) was able to induce first divisions in isolated microspores of pearl millet.

Phleum pratense L.

A small number of calluses were obtained from cultured anthers of timothy (Niizeki, 1977). Microscopic observation of the anthers revealed that various developmental stages had occurred in the microspores up to embryoid formation. The embryoids later developed cotyledons and roots but finally reverted to callus formation so that complete plantlets were not recovered.

Saccharum officinarum L.

The formation of callus tissue from cultured anthers of sugarcane has been reported by Moore and Maretzki (1974).

Secale species

Thomas and Wenzel (1975a) obtained diploid, triploid, and tetraploid plantlets from cultured anthers of rye, S. cereale L.; evidence was presented supporting the androgenetic origin of some of the plantlets. Androgenic haploid albino plants were later described by the same research team (Thomas et al., 1975; Wenzel et al., 1976a)

and green haploid rye plants have recently been obtained (Wenzel et al., 1977a).

Successful induction of androgenic haploids of rye has also been reported by Orlikowska (1977).

The cytological events occurring during the course of embryogenesis were recently reviewed by Sun (1978) and Sun et al. (1978).

The presence of multicellular pollen grains in cultured anthers of S. montanum Guss. has been reported by Zenkteler and Misiura (1974a).

Setaria italica Beauv.

Ban et al. (1971) obtained haploid plants from anther cultures of foxtail millet, S. italica. The authors noted that some of the haploids possessed a high degree of fertility.

Sorghum vulgare Pers. var. sudanense Hitchc. (S. sudanense Stapf)

Anther culture of sudan-grass was attempted by Herzky (1976) and Niizeki (1977) without success. The authors reported that callus tissue had not formed from any of the anthers.

X Triticosecale Wittmack

The production of haploid plants from cultured anthers of triticale was first reported by Chinese researchers (Wang et al., 1973b; Sun et al., 1974). Other successful reports include those by Bernard et al. (1976), Ono and Larter (1976), Bernard (1977), Orlikowska (1977), and Dorosiev et al. (1978). Pollen callus was observed in cultured triticale anthers by Heszky and Mesch (1976), but plantlets were not regenerated.

Haploid albino plantlets have regularly been obtained in triticale anther cultures, sometimes with a very high frequency (ca. 40%)

(Bernard, 1977).

Triticum species

The regeneration of haploid plantlets from cultured anthers of various cultivars of wheat, T. aestivum L., was first realized in 1973 by several research groups (Ouang et al., 1973; Picard and de Buyser, 1973; Wang et al., 1973a).

Successful results were also obtained by Craig (1974), Picard and de Buyser (1975, 1977), and Shimada and Makino (1975), using different wheat cultivars.

Ogura and Tsunewaki (1975) attempted to derive haploids from anther cultures of substitution lines of T. aestivum; calluses were obtained from anthers of various lines but haploid plantlets were not produced.

Considerable research on wheat anther culture has been performed by Chinese researchers, as may be seen by the impressive number of 30 communications which were presented at the Symposium on Anther Culture, held in Canton (China) in 1977 (see Proc. Symp. on Anther Culture, 1978a); various aspects of wheat embryogenesis were studied including for instance, the effect of the various constituents of culture media, the nature of the cytological events occurring during embryogenesis, the chromosomal variations and the ploidy levels in callus tissues and regenerants, the genotypic differences with regards to the frequency of embryoid induction, and the performance of androgenetic haploids in breeding experiments.

Fujii (1970) described the development of callus tissue from cultured anthers of T. aegilopoides Bal. and T. dicoccoides Koerm.; the origin of the callus was not investigated. He failed to induce

callogenesis in anthers of T. monococcum L., T. durum Desf. and T. spelta L.

Callus from microspore origin was obtained by Heszky and Mesch (1976) in T. durum, T. spelta, and T. ispahanicum Heslot.

Chu (1978) reported the recovery of albino haploid plants of T. dicoccoides from anthers cultured on N_6 medium (Chu et al., 1975) supplemented with 1 pmm 2,4-D, 2 ppm IAA, 1 ppm BA, 100 ppm myo-inositol, 0.5 ppm folic acid, 0.05 ppm biotin, and 8% sucrose. Plants were also obtained from anther culture of intergeneric hybrids between triticale (8x) and wheat; the ploidy of the regenerated plants remains to be determined. The author also mentioned that callus tissue was produced from cultured anthers of T. durum and from intergeneric hybrids between wheat and rye.

Triticum aestivum L. (T. vulgare Vill.) X Agropyron glaucum Roem. & Schult.

Haploid plants from the intergeneric hybrid between T. aestivum and A. glaucum were recovered from anther culture (Wang et al., 1975b).

Zea mays L.

Murakami et al. (1972) described the production of haploid calluses from anther cultures of corn; the differentiation of roots from the calluses was later observed. Callus tissue was also obtained by Opatrny et al. (1977); the origin of the tissue was not investigated. Heszky (1976) failed to induce callus formation in corn anthers.

Successful induction of androgenic haploids from corn anther cultures was recently reported by several Chinese research groups (Anonymous, 1978e,f; Ku et al., 1978a; Li and Ts'ui, 1978; Miao et al., 1978). Numerous studies have been undertaken in order to examine the various aspects of the phenomenon including: the nature of the cytological

events occurring during pollen embryogenesis and haploid callus formation (Ku et al., 1978b), the ploidy levels of the regenerants (Ku et al., 1978b), the progeny performance (Anonymous, 1978e), and the flowering and fruiting behavior of the haploids (Wang and Kuo, 1978).

Dicotyledoneae

Apocynaceae

Vinca rosea L. (Catharanthus roseus Don.)

At least two different haploid plants of V. rosea (n=x=8) have been regenerated from 31,000 cultured anthers (Abu-Mandoor and Czygan, cited in Nitzsche and Wenzel, 1977).

Asteraceae

Chrysanthemum species

First attempts to derive haploids from anther culture in this family were made by Watanabe et al. (1972). Callus tissue was induced on all cultured anthers of two diploid and six polyploid species of Chrysanthemum. The calluses, however, proved to be of somatic origin and no development of the microspores was observed.

Haplopappus gracilis (Nutt.) Gray

Watanabe et al. (1972) also mentioned obtaining 100% callus formation on cultured anthers of H. gracilis, but the ploidy of the callus cells was not ascertained.

Gerbera jamesonii Bolus

More recently, Preil et al. (1978) reported the regeneration of haploid plants from capitulum cultures of G. jamesonii. Callus formation

was observed mainly on the anthers in the tubular florets. The authors were successful in regenerating haploid plants from these calluses.

Betulaceae

Betula pendula Roth

Anther culture of European white birch, B. pendula, was undertaken by Huhtinen (cited in Nitzsche and Wenzel, 1977). Callus tissue was induced from anthers sown at the first pollen mitosis on MS medium supplemented with 0.1-1.0 ppm K and 1-10 ppm 2,4-D. Chromosome counts revealed that some of the calluses were haploid ($n=2x=28$), whereas others were diploid or triploid. The author concluded that the haploid calluses probably originated from the induced microspores. Unfortunately, shoots or roots could not be differentiated from these calluses.

Brassicaceae

Arabidopsis thaliana (L.) Heynh.

Haploid A. thaliana calluses and plants obtained via anther culture were described by Gresshoff and Doy (1972a). Meiotic anthers were cultured on a synthetic medium supplemented with a high auxin-low kinetin concentration favoring callus formation. Transfer of the calluses to a low auxin-high kinetin medium resulted in organogenesis; complete plantlets were regenerated, and a few flowered in the culture vessels. Chromosome counts performed on root tips of regenerated plantlets and callus cells indicated haploidy had taken place ($n=x=5$).

Avetisov (1976) has also reported the production of haploids during in vitro culture of A. thaliana anthers.

Brassica species

There exist in the literature numerous reports on anther culture in this economically very important genus.

Keller et al. (1975) demonstrated that plants could be regenerated from anther cultures of turnip rape, B. campestris L. Embryoids, ranging from globular to fully-differentiated stages, were produced in about 1% of the anthers. These had been cultured at the uninucleate or early binucleate stage on different media consisting of various modifications of the B5 medium. Sucrose concentration was found to play an important role in embryoid induction. Levels below 6% did not result in embryoid production. According to the authors, high sucrose concentration not only inhibited the growth of somatic tissues, but specifically induced pollen divisions. Although none of the plants obtained were haploid, the authors provided sufficient evidence to demonstrate that the anther-derived plants were of pollen origin. However, haploid plantlets were obtained from anther cultures of B. campestris by Chinese researchers (Anonymous, 1975).

Sunderland et al. (1971) was unsuccessful in inducing the formation of pollen embryoids or pollen calluses from cultured anthers of white mustard, B. hirta Moench (Sinapis alba L.). From cultured anthers of B. juncea Coss., he obtained only somatic callus tissue from which root development occurred.

Pollen divisions in cultured anthers of B. napus L. were first described by Thomas et al. (1974). In a subsequent report, Thomas and Wenzel (1975b) observed embryogenesis in anthers of B. napus cultured on a modified LS medium supplemented with various hormones. One embryoid was produced from pollen which had been separated from an anther during

incubation. Callus cultures were initiated from this embryoid and a haploid plantlet (n=19) was regenerated. Multicellular structures were also obtained from the culture of isolated microspores but no further development was noted (Thomas et al., 1976). Subsequent studies brought about the recovery of 17 haploid and 48 diploid plants (Wenzel et al., 1977b).

Although Keller and Armstrong (1977) failed to detect haploids in 31 anther-derived B. napus plants, observations on embryoid development in the cultured anthers provided evidence for the microspore origin of these plants. The authors later reported (Keller and Armstrong, 1978) that haploids of B. napus could be recovered from anthers submitted to high temperature treatments (30, 35, and 40°C).

Elmsheuser and Neumann (1978) have recently reported that their attempts to trigger pollen embryogenesis in B. napus, using cold treatments and activated charcoal were unsuccessful.

Anther-derived doubled-haploid lines of B. napus are now available and under field tests at Saskatoon (Stringham, cited in Keller and Stringham, 1978). Spontaneous amphidiploids of pollen origin have also been evaluated in a breeding program aimed at developing low glucosinolate lines (Hoffmann et al., 1976).

The induction of haploid plants from cultured anthers of B. oleracea L. and the hybrid B. oleracea X B. alboglabra was first reported by Kameya and Hinata (1970). Callus tissue was produced on almost mature anthers cultured on a modified Nitsch medium (1951) supplemented with various growth regulators; plantlets were subsequently regenerated from these calluses. Although the chromosome number of the callus cells varied (9, 10-17, and 18 chromosomes), all the plantlets

were haploid (n=x=9). The authors were also able to induce the formation of cell clusters from isolated mature pollen grains of B. oleracea and B. oleracea X B. alboglabra. However, no shoot regeneration took place on the callus masses. The other species tested by the authors, namely, B. pekinensis Rupr., B. chinensis L., and B. alboglabra, did not show any response.

Somatic callus formation in cultured anthers of three B. oleracea varieties, namely, var. acephala DC. (common kale), var. botrytis L. (cauliflower), and var. gemmifera Zenker (brussels sprouts), was described by Sunderland et al. (1971). Pollen divisions were not observed.

Unsuccessful attempts to induce androgenesis in cabbage, B. oleracea var. capitata L., were reported by Miszke and Skucińska (1975). A few multicellular structures were later observed by the authors in the pollen sacs of cultured cabbage anthers (Skucińska and Miszke, 1978).

Anther culture studies in broccoli, B. oleracea var. botrytis, were recently reported by Quazi (1978). Callus tissue and later plants developed from anthers cultured on a synthetic medium supplemented with various hormones and organic addenda. Chromosome counts on root tips from a rhizogenic callus revealed that the roots were tetraploid (2n=4x=36), whereas the donor plants were diploid.

The recovery of haploid plantlets from cultured anthers of B. chinensis and B. oleracea was recently obtained by Chung et al. (1978); Teng and Kuo (1978) also reported the successful induction of androgenetic haploids of B. pekinensis.

Raphanus sativus L.

Anther culture in R. sativus has not been successful (Sunderland et al., 1971).

Chenopodiaceae

Beta vulgaris L.

First attempts to culture anthers of sugar beet, B. vulgaris, were described by Banba and Tanabe (1972). Callus tissue was obtained from the cultured anthers but the ploidy of the cells was not reported.

A few years later, the successful induction of haploid plantlets from anther cultures in this species was realized by a Chinese research group (Pao et al., 1978). The authors mentioned that induction of plantlet regeneration presented some difficulty and none of the few plantlets obtained (four from a tetraploid cultivar and three from a diploid cultivar) survived transfer to soil.

Convolvulaceae

Ipomoea nil Roth (Pharbitis nil Choisy)

The induction of haploid callus from anther cultures of morning glory, I. nil, was reported by Sangwan and Norreel (1975a). Upon transfer to a low hormone madium, a few calluses were induced to differentiate embryos and plantlets. The ploidy of these structures was not reported; however, cytological examination of the embryogenic calluses revealed that the cells were nearly all haploid, more rarely diploid or triploid.

Euphorbiaceae

Hevea brasiliensis Muell. Arg.

Recently, Chen et al. (1978a,b) reported the regeneration of haploid plantlets from anther cultures of an economically important species, H. brasiliensis, the caoutchouc tree. Embryoids were obtained

20 days after anthers had been cultured, mostly at the uninucleate stage on MS medium supplemented with 1 ppm K, 1 ppm 2,4-D, and 3% sucrose. The subsequent transfer of these embryoids into various culture media was found necessary to further their development. Cytological examination of the embryoids revealed that they were haploid (n=x=18). Numerous aneuploid cells (<26, 27,>28 chromosomes) were scored in the root tips of the plantlets derived from these embryoids, but diploid cells were not observed (Chen et al., 1978b).

Similar results were obtained by a different Chinese research group (Anonymous, 1978c).

Fabaceae

Several attempts have been made to induce androgenetic haploid plants in this economically very important family.

Arachis hypogaea L.

Anther culture of peanut was undertaken by Martin and Rabéchault (1976). Haploid cells were rarely observed in the callus tissue obtained from the cultured anthers and most cells exhibited the diploid number of chromosomes.

Glycine max (L.) Merr.

Profuse callus developed from cultured soybean anthers on a variety of culture media (Ivers et al., 1974). Cytological examination of the roots that differentiated from the callus tissue revealed that the cells were diploid, leading the authors to believe that the tissue was of somatic origin.

Chien et al. (1978) recently reported observing multicellular pollen grains in soybean anthers cultured on a modified B5 medium.

Lotus species

Anther culture has been attempted in several Lotus species and hybrids (Table III). The induction of pollen mitoses leading to the development of pollen callus and/or pollen embryoids has not been successfully triggered. The occurrence of haploid cells in the callus tissue derived from the cultured anthers was not reported and the plantlets regenerated from these calluses proved to be either tetraploid (Niizeki, 1971; MacDonald, 1974) or octoploid (Niizeki, 1971; Niizeki and Grant, 1971).

Medicago sativa L.

Callus tissue was obtained from alfalfa anthers cultured at various stages of development (Saunders and Bingham, 1972). Histological studies indicated that, in most cases, the callus arose from the connective cells.

Niizeki and Kita (1973) and Niizeki (1977) reported the recovery of callus from cultured anthers of alfalfa, but the callus tissue appeared to be of somatic origin. Organ regeneration did not proceed from these calluses.

Attempts to culture mature anthers of two alfalfa cultivars treated with Ethrel, an ethylene-releasing compound, proved unsuccessful (MacDonald, 1974; MacDonald and Grant, 1974b). The authors concluded that the nuclear bodies observed in the pollen grains were micronuclei originating from Ethrel-induced abnormal mitoses. The ploidy of the callus tissue obtained from the cultured anthers was not reported.

Phaseolus vulgaris L.

Peters et al. (1977) reported the presence of haploid cells in

TABLE III. *Lotus* species and hybrids used in anther culture studies

Species	References
L. alpinus Schleich. (2x)	MacDonald, 1974; Macdonald and Grant, 1974a.
L. alpinus Schleich. (4x)	Niizeki, 1971; MacDonald, 1974; MacDonald and Grant, 1974a.
L. caucasius Kupr.	Niizeki, 1971; Niizeki and Grant, 1971; MacDonald, 1974; MacDonald and Grant, 1974a, Niizeki, 1977.
L. corniculatus L.	Niizeki, 1971; MacDonald, 1974; MacDonald and Grant, 1974a; Tomes, 1976.
cv. Empire	Niizeki, 1971; Niizeki and Grant, 1971; Niizeki and Kita, 1973; MacDonald, 1974; MacDonald and Grant, 1974a; Niizeki, 1977.
cv. Maitland	MacDonald, 1974; MacDonald and Grant, 1974a.
cv. M. C. H.	MacDonald, 1974; MacDonald and Grant, 1974a.
cv. Viking	Niizeki, 1971; Niizeki and Grant, 1971; MacDonald, 1974; MacDonald and Grant, 1974a; Niizeki, 1977.
var. *crassifolius*	Niizeki, 1971; Niizeki, 1977.
L. cruentus Court.	MacDonald, 1974; MacDonald and Grant, 1974a.
L. divaricatus Boiss.	MacDonald, 1974; MacDonald and Grant, 1974a.
L. hispidus Desf.	Niizeki, 1971; MacDonald, 1974; MacDonald and Grant, 1974a.
L. japonicus (Regel) Larsen (2x and 4x)	MacDonald, 1974; MacDonald and Grant, 1974a.
L. krylovii Schischk. et Serg.	MacDonald, 1974; MacDonald and Grant, 1974a.

Continued...

TABLE III. (Continued)

Species	References
L. pedunculatus Cav. (2x and 4x)	MacDonald, 1974; MacDonald and Grant, 1974a.
L. peregrinus L.	Niizeki, 1971.
L. sulphureus Boiss.	MacDonald, 1974; MacDonald and Grant, 1974a.
L. tenuis Waldst. et Kit. (2x)	Niizeki, 1971; MacDonald, 1974; MacDonald and Grant, 1974a,b.
L. tenuis Waldst. et Kit. (4x)	Niizeki, 1971; MacDonald, 1974; MacDonald and Grant, 1974a.
(L. japonicus (Regel) Larsen X L. alpinus Schleich.)^2	MacDonald, 1974; MacDonald and Grant, 1974a.
(L. japonicus (Regel) Larsen X L. alpinus Schleich.)^2 X L. corniculatus L.	MacDonald, 1974; MacDonald and Grant, 1974a.
(L. krylovii Schischk. et Serg. X L. alpinus Schleich.)^2	MacDonald, 1974; MacDonald and Grant, 1974a.

calluses derived from cultured anthers of kidney beans. Veliky and Martin's culture medium (1970), supplemented with either 2,4-D (1 ppm) alone or NAA (1 ppm), IAA (2 ppm), and K (0.2 ppm), was utilized in these studies.

Trifolium pratense L.

The formation of callus from cultured anthers of red clover was observed by Heszky (1976). He also reported the development of roots in the cultures.

Vicia faba L.

Failure to initiate pollen divisions and callus formation from cultured anthers of two broad bean cultivars was reported by Keller (1974).

Geraniaceae

Pelargonium hortorum Bailey

The regeneration of plants from anther culture of fish geranium, P. hortorum, was first reported by Abo El-Nil and Hildebrandt (1971). Haploids were later obtained from anthers cultured at the uninucleate stage on a modified White's medium (1943) supplemented with 2-2.5 ppm NAA, 2.5 ppm K, 150 mg/l CM, and 2% sucrose. Upon repeated divisions, the microspores gave rise to a callus tissue which was induced to differentiate plantlets (Abo El-Nil and Hildebrandt, 1973). The authors noted that the induced pollen grains never developed directly into embryoids.

Gesneriaceae

Saintpaulia ionantha Wendl.

Haploid plants were recovered from cultured anthers of one member of this family, S. ionantha, the African violet (Hughes et al., 1975). Preliminary studies indicated that late uninucleate microspores, that is, approaching or undergoing the first mitotic division following meiosis, were induced into division. All regenerated plantlets showed a haploid number of chromosomes (n=x=14).

Hippocastanaceae

Aesculus hippocástanum L.

Radojević (1978) described the induction of pollen embryoids and the regeneration of haploid plantlets from cultured anthers of the horse-chestnut tree. These results were obtained with anthers sown at the uninucleate stage on a solid MS medium supplemented with 2% sucrose, various vitamins, organic addenda, and growth regulators. The auxin 2,4-D was found essential for embryogenesis, as no embryoids were observed in anthers inoculated on media lacking the growth regulator.

Linaceae

Linum usitatissimum L.

Anther culture of two cultivars of flax has resulted in the formation of calluses on which buds developed (Pao et al., 1978). Cytological examination of the callus cells or the buds was not reported.

Malvaceae

Gossypium species

The formation of haploid pollen callus was first reported in Gossypium sp. by Nitsch (cited in Sharp et al., 1972a,b). In contrast, Haccius (1972) was unsuccessful in inducing embryogenesis in cultured anthers of levant cotton, G. herbaceum L.

Anthers of G. anomalum Wawra & Peyr. cultured at the quartet stage on Nitsch's medium (1974a) supplemented with different addenda did not produce callus or plantlet formation (Merriam and Scowcroft, 1976).

Chinese researchers have obtained calluses from cultured anthers of upland cotton, G. hirsutum L. (Anonymous, 1978a; Hsi and Wu, 1978). The best response was elicited from anthers given a cold pretreatment and sown at the mid- or late-uninucleate stage on a synthetic medium supplemented with 0.5 ppm 2,4-D, 0.5 ppm IAA, 2 to 3 ppm NAA, and 2 ppm K. The authors reported the addition of 0.5 to 1 ppm GA to also be beneficial. Organogenesis proceeded after the calluses were transferred onto a differentiating medium. Roots, embryo-, shoot-, and leaf-like structures were noted. The origin of the callus tissue remains to be determined. Similar results were described by Hu (1978).

Recently, Sharma and Vijayakumar (1978) observed whitish callus tissue growing from cultured anthers of G. hirsutum and G. herbaceum. The anthers were sown at the quartet stage on MS medium supplemented with 0.05 ppm IAA, 0.1 ppm K, and 1% malt extract. Cytological examination of the callus tissue showed that it had originated from microspores. Organogenesis was not reported.

Barrow et al. (1978) reported the initiation and subculture of

callus tissue from anthers of G. hirsutum and sea-island cotton, G. barbadense L., cultured on various media. Cytological examination of a small number of fast growing G. hirsutum cell suspensions derived from anther calluses, revealed that 83% of the cells were haploid (n=2x=26).

Papaveraceae

Papaver bracteatum Lindl.

Haploid poppy plants have been regenerated from microspores by Corduan, and Abu-Mandoor and Czygan (cited in Nitzsche and Wenzel, 1977).

Primulaceae

Cyclamen persicum Mill.

Cultured anthers of cyclamen failed to show any response (Haccius, 1972).

Ranunculaceae

Anemone species

Sunderland et al., (1971) attempted to induce pollen embryoids or pollen callus in anthers of the poppy anemone, A. coronaria L., cultured before or after the first pollen mitosis. Pollen grains survived as long as six months in culture, but failed to show any response. Diploid callus formation was also extremely rare.

Multicellular proembryonic structures were later observed in cultured anthers of A. coronaria, which exhibited pollen dimorphism (Sunderland and Roberts, 1975). The authors determined that only the aberrant microspores were induced into division.

The development of embryos from cultured anthers of four Anemone species, A. virginiana L., A. rupicula Camb., A. multifida Poir.,

and the meadow anemony, A. canadensis L., was reported by Johansson and Eriksson (1977). The cotyledonary embryos were recovered from anthers sown on Nitsch and Nitsch's basic medium (1969) supplemented with various concentrations of activated charcoal and incubated preferably in darkness at 25°C. Development of these embryos into plantlets was not reported however.

Helleborus species

Multicellular pollen grains were observed in anthers of two Helleborus species, the christmas-rose, H. niger L., and H. foetidus L., cultured at the uninucleate stage on MS medium supplemented with 3% sucrose and various combinations of hormones (Zenkteler and Misiura, 1974a; Zenkteler et al., 1975). Further development of these grains was not reported.

Paeonia species

There exist in the literature several reports on anther culture in the genus Paeonia.

First attempts were made by Sunderland et al. (1971) with P. daurica Anders., P. emodi Wall., P. mlokosewitschi Lomák. var. tenuifolia and various hybrids. Four-celled grains were observed in P. mlokosewitschi, up to six cells in P. daurica, and eight cells in P. emodi. Anthers were cultured on various media either at the uninucleate stage or at the first pollen mitosis. According to the authors, multicellular grain formation was favored by MS medium supplemented with either a low NAA or 2,4-D concentration (0.1 ppm) and a high concentration of kinetin (5 ppm). Light regime seemed unimportant. No further growth was reported. A few pollen embryoids were later

observed in two peony species, P. decora Anders., and P. triternata Pall. (Sunderland et al., 1973a, 1975). Embryoid formation was noted by Sunderland (1974) in anthers of P. hybrida Pall. cultured on a variety of media. The course of development of these Paeonia pollen embryoids was studied by Haccius et al. (1975). Plantlet development was later reported by Roberts and Sunderland (1977).

Multinuclear and multicellular pollen grains were obtained by Zenkteler and Misiura (1974a) and Zenkteler et al. (1975) in anthers of several Paeonia species, P. lutea Delavay ex Franck. var. superba, P. officinalis L., P. tenuifolia L., and the tree peony, P. suffruticosa Haw., cultured at the uninucleate stage on MS medium supplemented with 3% sucrose and various growth regulators.

Callus formation, presumably from microspores, occurred in cultured anthers of P. lactiflora Pall. (P. albiflora Pall.) as reported by Harn (1976). Different division patterns, which resulted in the formation of various types of multinucleate and multicellular pollen grains, were observed in the microspores.

Ono and Tsukida (1978) successfully induced the formation of haploid callus ($n=x=5$) from cultured anthers of P. lactiflora Pall. f. hortensis. The haploid calluses were recovered from anthers sown on MS media supplemented with either 15% CM or 2 ppm NAA, 1 ppm 2,4-D, and 0.1 ppm K.

Ranunculus sceleratus L.

Profuse callusing was obtained from mature anthers of cursed crowfoot cultured on White's medium (1943) supplemented with 10% CM, 0.5 to 2 ppm 2,4-D, and 2% sucrose (Konar and Nataraja, 1965). Histological studies of the cultured material revealed that the callus

originated from the connective tissue of the anther.

Rosaceae

Fragaria chiloensis Duchesne var. ananassa Bailey

Anther culture of the cultivated strawberry was unsuccessful in producing haploids (Fowler et al., 1971). The callus tissue that formed on the anthers proved to be of somatic origin.

Devreux et al. (1975) recovered diploid and tetraploid plantlets from cultured strawberry anthers.

Malus species

Callus formation from cultured anthers of apple trees was first reported by Nakayama et al. (1971, 1972).

Attempts by Zenktelér and Misiura (1974a) to induce pollen divisions in cultured anthers of Siberian crab-apple, M. baccata Borkh., and paradise apple, M. sylvestris Mill. var. paradisiaca Bailey (M. pumila Mill.), were unsuccessful.

A few years later, Milewska-Pawliczuk and Kubicki (1977) reported the development of embryoids from anther cultures of common apple, M. domestica Baumg. cv. Jonathan. The embryoids which were induced most successfully from microspores cultured at the uninucleate stage developed up to the torpedo stage but degenerated soon after. Plantlets were not recovered from these cultures.

Prunus species

There are several reports in the literature on anther culture in the genus Prunus. Harn and Kim (1972) succeeded in inducing callus from anthers of the common apricot P. armeniaca L. According to the authors,

the callus tissue was probably of microspore origin.

Callus tissue also developed from cultured anthers of the peach tree, P. persica Batsch, and the almond tree, P. amygdalus Batsch (Michellon et al., 1974); haploid callus cells were observed by the authors in P. persica.

Pollen embryoids were detected in anthers of the sweet cherry tree, P. avium L., cultured on MS medium supplemented with 1 ppm BAP, 1 ppm IAA, and 12% sucrose (Zenkteler and Misiura, 1974a; Zenkteler et al., 1975). Multicellular structures of microspores origin were obtained by Jordan (1974; 1975) in cultured anthers of the same species.

Pyrus sp.

Microspore divisions in cultured anthers of a Pyrus species were observed by Jordan (1975).

Rosa sp.

Attempts to induce pollen divisions in cultured anthers of roses, Rosa sp., have recently been reported (Jain and Guha-Mukherjee, 1978). The authors observed callus formation from the anthers but the origin of this callus tissue was not investigated.

Rubiaceae

Coffea arabica L.

Sharp et al. (1973) successfully induced callus proliferation from anthers of two cultivars of coffee cultured on MS medium supplemented with various concentrations of auxins and cytokinins. The callus cells showed the dihaploid ($2x=22$), or in some instances, the haploid ($x=11$) number of chromosomes. However, the differentiation of haploid plantlets

was not reported.

Rutaceae

Citrus species

Anther culture of two Citrus species, C. medica L., the citron tree, and C. limon L., the lemon tree, was attempted by Drira and Benbadis (1975). Callus tissue was obtained from both species; according to the authors, the presence of glutamine in the culture medium was indispensable for the formation of the callus. In the case of citron, the callus originated from the connective tissue; in C. limon the divisions which were observed in the cultured microspores lead to the formation of multinuclear masses and androgenetic calluses. Determination of the ploidy of the callus cells was not reported.

Salicaceae

Populus nigra L.

The recovery of haploid plantlets from black poplar anthers cultured on a synthetic medium was reported by Wang et al. (1975a).

Similar successful results were recently obtained by Lu (C.-H.) et al. (1978) with four Populus species and six interspecific hybrids (see Table II); the authors observed haploidy or near haploidy in root tips of the regenerated plantlets.

Saxifragaceae

Ribes species

Utilizing Murashige and Skoog's medium (1962) supplemented with various combinations of hormones and relatively high sucrose levels

(6% and 9%), Zenkteler and Misiura (1974a) were able to induce multicellular pollen grains in the cultured anthers of European black currant, R. nigrum L. Anthers of a different species, R. sanguineum Pursh var. atrorubens Hort., cultured on the same media failed to show a response.

A few microspore divisions were also observed in anthers of a Ribes species cultured on Nitsch's medium (1972) supplemented with 1 ppm NAA and 1 ppm BAP (Jordan, 1975). Further development of the induced pollen grains was not reported.

Scrophulariaceae

Digitalis species

Sunderland et al. (1971) reported rapid diploid callus formation on cultured anthers of common foxglove, D. purpurea L.

A few years later, Corduan and Spix (1975) reported the induction of haploid callus formation and the regeneration of plants from anthers of D. purpurea L. Production of callus was obtained on several basal media supplemented with various amounts of 2,4-D. Although the callus tissue was haploid, most regenerated plants were euploids. Cytological observations demonstrated that regeneration started from haploid callus, leading to intermediate degrees of ploidy and finally to diploid plants. Five haploid ($n=x=28$) plantlets were recovered but did not survive transfer to soil.

More recently, Corduan was able to regenerate plants from the generative cells of Grecian foxglove, D. lanata Ehrh. (cited in Nitzsche and Wenzel, 1977).

Linaria maroccana Hook. f.

The formation of a white and very fragile callus tissue from cultured anthers of a Moroccan species of toadflax has recently been reported by Kumar and Tyagi (1978). Pollen divisions up to a seven-celled stage were observed but insufficient numbers of dividing callus cells prevented the authors from assessing the ploidy level. They stated, however, that the callus tissue was haploid. Morphogenesis was not observed in the cultures.

Solanaceae

Atropa belladonna L.

The production of haploid plants (n=36) from anther cultures of belladonna was first realized by Zenkteler (1971). Anthers were inoculated at the uninucleate stage on a solid LS medium supplemented with 4 ppm K and 2 ppm IAA.

Successful results were also obtained by Narayanaswamy and George (1972) using a slightly different medium (MS) supplemented with various growth regulators and organic addenda. Studies by Rashid and Street (1973) indicated that embryogenesis was most often initiated by a symmetrical division in the uninucleate microspore forming two nuclei which stained to the same extent.

Multicellular structures were noted in cultures of isolated pollen mother cells of A. belladonna (Bajaj, 1974). Further development was not reported however.

Capsicum frutescens L. (Capsicum annum L.)

In cultured anthers of red pepper, Sunderland et al. (1972) observed the development of a few pollen embryoids; these did not

develop further under the cultural conditions assayed. However, the recovery of androgenic pepper plants was independently obtained the following year by three research groups (George and Narayanswamy, 1973; Kuo et al., 1973; Wang et al., 1973b).

Novák (1974) reported the presence of haploid cells in calluses derived from cultured anthers of red pepper but the plantlets did not differentiate in the cultures. Ethrel, an ethylene-releasing compound, did not induce embryoid formation in cultures of mature pepper anthers (MacDonald and Grant, 1973; 1947b).

Recently, Wang et al. (1978a,b) described the recovery of androgenetic haploid peppers which they were able to diploidize.

Datura species

Embryo-like structures growing from cultured mature anthers of D. innoxia Mill. were observed by Guha and Maheshwari (1964); these structures were later determined as having originated from the pollen grains (Guha and Maheshwari, 1966, 1967). Subsequently the plantlets that evolved from the embryoids were shown to be haploid. This constituted the first example of in vitro embryogenesis occurring in the male gametophyte.

Various aspects of anther and pollen culture in D. innoxia have been examined; for instance, the effect of the different components of the culture medium (Nitsch and Nitsch, 1970; Sopory and Maheshwari, 1972, 1973, 1976a,b; Forche and Neumann, 1977), the importance of the developmental stage of the microspores at the time of excision (Engvild et al., 1972; Sopory and Maheshwari, 1972, 1976a), the influence of pretreatments (Nitsch and Norreel, 1973; Sunderland et al., 1973b; Sunderland and Roberts, 1976; Forche and Neumann, 1977; Sangwan-Norreel, 1977), the

nature of the cytological events occurring during the induction of embryogenesis and the following developmental stages (Nitsch and Nitsch, 1970; Norreel, 1970; Geier and Kohlenbach, 1973; Collins et al.,1974a; Dunwell and Sunderland, 1974a, 1976a,b,c; Nitsch, 1974b; Sunderland, 1974; Sunderland et al., 1974), and the ploidy of the callus cells and the regenerated plantlets (Engvild et al., 1972; Geier and Kohlenbach, 1973; Collins et al., 1974a).

Haploid androgenic D. innoxia plants were reported by several investigators (see Table II).

Attempts by Laneri and Chirilà (1976) to culture isolated microspores of D. innoxia were partially successful; embryogenesis was induced but maturation of the embryoids into plantlets did not proceed. Sangwan-Norreel (1977) recently described a method to improve yields of androgenic embryoids from cultured isolated microspores of D. innoxia, but plantlet maturation was not reported.

Kohlenbach and Geier (1972), Nitsch (1972), and Geier and Kohlenbach (1973) successfully induced haploid plants of D. meteloides Dunal.

The recovery of pollen embryoids from cultured anthers of D. stramonium L. was reported by Guha and Maheshwari (1967).

Androgenic haploid plants of D. wrightii Regel were obtained by Kohlenbach and Geier (1972) and similar successful results were described by Nitsch (1972) with D. muricata Link anther culture.

Haploid plants from D. metel L. arising from cultured anthers have been reported by several researchers (Chandy and Narayanaswamy, 1971; Narayanaswamy and Chandy, 1971; Iyer and Raina, 1972; Nitsch, 1972).

Unsuccessful attempts to produce haploids from anthers of

D. quercifolia Godr., D. tatula L., D. stramonium, and D. wrightii were reported by Nitsch (1972).

Hyoscyamus species

The regeneration of androgenic plants of H. albus L., H. niger L., and H. pusillus L. was described by Raghavan (1975). The anthers were cultured at the uninucleate stage on Nitsch and Nitsch's medium (1969) without hormonal supplementation.

Corduan (1975) also reported the differentiation of haploid, as well as diploid, plants which originated from cultured anthers of H. niger. The development of plants from isolated H. niger microspores cultured in liquid medium was observed by Wernicke and Kohlenbach (1977).

The mode of origin of pollen embryoids and pollen calluses of H. niger has recently been studied by Raghavan (1976, 1978) and Sunderland et al. (1977) and evidence is presented for the participation of both nuclei (vegetative and generative) in the development of multicellular structures.

Lycium halimifolium Mill.

Zenkteler (1972) reported the recovery of haploid green and albino plantlets from anther cultures of L. halimifolium. The embryogenic process took place in anthers sown at the uninucleate stage on LS medium supplemented with 1 ppm K and 0.5 ppm IAA.

Lycopersicon species

Haploid callus from cultured anthers of tomato, L. esculentum Mill., was obtained by Sharp et al. (1971). The authors were later able to grow haploid tomato clones from isolated microspores using nurse cultures (Sharp et al., 1972b).

Gresshoff and Doy (1972b) reported the differentiation of haploid tomato plantlets from anther-derived callus tissue initiated on several culture media. Haploid calluses were also obtained from cultured anthers of L. peruvianum Mill. and L. pimpinellifolium Mill.

Following unfruitful attempts to produce haploid plants from cultured anthers, Debergh and Nitsch (1973) succeeded in inducing embryogenesis in isolated microspores; developmental stages up to the cotyledonary stage were observed in the two species cultured, L. esculentum and L. pimpinellifolium. Further development of these embryoids was not reported.

Unsuccessful anther culture studies in L. esculentum have recently been reported by Dao et al. (1976) and Heszky (1976).

Devreux et al. (1976) and Sree Ramulu et al. (1976b) were able to trigger the formation of callus from cultured anthers of L. peruvianum, a close relative of the tomato. Diploid and tetraploid plantlets were recovered but haploid plants were not produced from any of the genotypes studied.

Nicotiana species

Since Bourgin and Nitsch's report (1967) on the recovery of androgenetic haploid plants from tobacco, N. tabacum L., anther and pollen culture in this species has successfully been induced by a great number of investigators (see Table II) and is now regarded as a model system of androgenesis. Different aspects of tobacco anther and pollen culture have been researched; these include studies on the cytological, ultrastructural, and biochemical events occurring during embryogenesis (Nitsch, 1969; Nitsch and Nitsch, 1970; Norreel, 1970; Dunwell et al., 1971; Sunderland and Wicks, 1971; Vazart, 1971; Dunwell and Sunderland, 1972;

Pelletier and Ilami, 1972; Bhojwani et al., 1973; Pelletier, 1973; Vazart, 1973; Dunwell and Sunderland, 1974b,c, 1975; Rashid and Street, 1974; Horner and Street, 1978), the effect of the culture medium composition and the cultural conditions (Nitsch and Nitsch, 1970; Sunderland and Dunwell, 1971; Corduan, 1973; Dunwell and Perry, 1973; Anagnostakis, 1974; Kasperbauer and Collins, 1974; Dunwell and Roberts, 1975; Dunwell, 1976; Wernicke and Kohlenbach, 1976; Forche and Neumann, 1977; Phillips and Collins, 1977; Anonymous, 1978i), the effect of temperature pretreatments (Sunderland and Roberts, 1977a,b, 1979; Lu, 1978a,b), and the determination of the most appropriate stage of development for embryoid induction (Sunderland and Wicks, 1969; Nitsch, 1972; Norreel, 1973; Mii, 1976). Anther and pollen culture in this species has also generated cytological studies of callus tissue and regenerants (Collins et al., 1972; Niizeki, 1973, 1974; Engvild, 1974; Gorenflot et al., 1975; Novák and Vyskot, 1975), as well as cytogenetical (Mattingly and Collins, 1974; Tsikov et al., 1974; Niizeki, 1975; Niizeki and Kita, 1975a,b, 1977; Sarvella et al., 1976), mutation (Nitsch et al., 1969; Nilsson-Tillgren and Wettstein-Knowles, 1970; Vagera et al., 1976; Vagera, 1978), and breeding research (Collins et al., 1974b; Collins and Legg, 1975; Burk and Matzinger, 1976; Anonymous, 1978g,h; Arcia et al., 1978; Zagorska et al., 1978).

The very interesting results obtained with N. tabacum prompted several investigators to attempt the production of haploids in other Nicotiana species. So far, haploid plantlets or plants have been recovered from cultured anthers of 13 species and interspecific hybrids (see Table II).

Failure to induce androgenetic haploids from cultured anthers has been recorded for the following species: N. acuminata Hook. (Nitsch, 1969,

1972), N. bigelovii Wats.(Nitsch, 1972; Tomes and Collins, 1976), N. bonariensis Lehm. (Nitsch, 1969, 1972; Tomes and Collins, 1976), N. eastii Kostoff (Tomes and Collins, 1976), N. excelsior (Nitsch, 1972), N. forgetiana Sander (Nitsch, 1972; Tomes and Collins, 1976), N. glauca Graham (Nitsch, 1969, 1972; Tomes and Collins, 1976), N. glutinosa L. (2x) (Nitsch, 1969), N. goodspeedii (Nitsch, 1972), N. gossei Domin (Tomes and Collins, 1976), N. langsdorffii Schrank (Nitsch, 1969, 1972; Tomes and Collins, 1976), N. longiflora Cav. (Nitsch, 1969, 1972; Tomes and Collins, 1976), N. megalosiphon Heurck & Muell. (Tomes and Collins, 1976), N. miersii Remy (Tomes and Collins, 1976), N. nudicaulis Wats. (Tomes and Collins, 1976), N. plumbaginifolia Viv. (Tomes and Collins, 1976), N. repanda Willd. ex Lehm.(Nitsch, 1969, 1972; Tomes and Collins, 1976), N. simulans N. T. Burb. (Nitsch, 1972), N. stocktonii Brandegee (Tomes and Collins, 1976), N. suaveolens Lehm. (Nitsch, 1969, 1972; Tomes and Collins, 1976), N. triginophylla Dun. (Tomes and Collins, 1976), N. undulata Ruiz & Pavon (Tomes and Collins, 1976), and N. velutina (Nitsch, 1972).

Haploid callus was reported in cultured anthers of N. suaveolens X N. langsdorffii (Guo, 1972), but organogenesis was not described by the author. Unsuccessful results were also obtained in anther cultures of N. glauca X N. langsdorffii and N. suaveolens X N. glauca (Nitsch, 1969), and N. sylvestris X N. tabacum and N. langsdorffii X N. glauca (Bourgin and Nitsch, 1967).

Petunia species

From cultured anthers of an interspecific hybrid between P. axillaris BSP and P. hybrida Vilm., Raquin and Pilet (1972) obtained

one haploid as well as several diploid and triploid plants. Cytological studies conducted in order to determine the origin of the non-haploid plants provided evidence for an androgenetic provenance.

Engvild (1973) also reported the recovery of androgenic triploid plantlets from cultured anthers of P. axillaris. He proposed that this species produced predominantly triploids presumably because plantlets developed from older binucleate pollen grains in which DNA synthesis in the generative nucleus was already completed. Plantlets of P. axillaris were also observed by Swamy and Chacko (1973) in anther cultures; the ploidy of the regenerants was not reported.

Haploid callus (Raquin and Pilet, 1972) and haploid, diploid, and triploid androgenetic plants (Maizonnier, 1973; Raquin, 1973; Wagner and Hess, 1974) were obtained from cultured anthers of P. hybrida. Unsuccessful results were reported by Bernard (1971).

Multicellular structures (Binding, 1972) and haploid plants (Sangwan and Norreel, 1975b) have been reported from the culture of isolated microspores of P. hybrida.

Scopolia species

The development of embryoids in anther cultures of three Scopolia species, S. carniolica Jacq., S. lurida Dun., and S. physaloides Dun., was observed by Wernicke and Kohlenbach (1975). Haploid plants were recovered only from the latter species, S. physaloides.

Solanum species

The differentiation of haploid plants from calluses derived from cultured anthers of S. nigrum L. was described by Harn (1971; 1972a,b). The anthers were sown at various stages of development on MS medium

supplemented with different growth regulators. Similar findings were also obtained by Irikura (1975).

Irikura and Sakaguchi (1972) were successful in inducing haploid plants (n=12) of S. verrucosum Schlecht. from microspores cultured at the first pollen mitosis on various media. Irikura (1975) later confirmed these results.

The successful production of several pollen embryoids and one haploid plantlet in the cultivated potato, S. tuberosum L., was reported by Dunwell and Sunderland (1973). Their findings were duplicated by Irikura (1975).

Foroughi-Wehr et al. (1977) produced monohaploids (n=x=12) from dihaploid (n=2x=24) S. tuberosum obtained parthenogenetically by pollination with S. phureja Juz. & Buk. (Hougas et al., 1958). Similar results were obtained by Sopory et al. (1978); the authors were able to improve embryoid yields, and many of these embryoids were monohaploid. The addition of 0.5% activated charcoal, 4 X 10^{-6} M BAP, and 6% sucrose, was found necessary to produce an optimal response.

Several authors have failed to induce androgenic plants in a number of potato cultivars (Irikura and Sakaguchi, 1972; Kohlenbach and Geier, 1972; Labib and Melchers, 1972; Zenkteler and Misiura, 1974a; Sinha et al., 1976).

Pollen callus formation has been induced in cultured anthers of the eggplant, S. melongena L. (Raina and Iyer, 1973). Although cytological studies of the callus revealed a haploid condition (n=12), all the regenerated plants were diploid. The authors believed that polyploidization resulted from chromosome duplication occurring during callus growth. Haploid androgenic S. melongena plants were obtained by a Chinese research

group (Anonymous, 1978d,j). The plants were regenerated either directly from embryoids or via a callus phase; several culture media were utilized. Similar successful results were recently reported by Isouard et al. (1979).

Zenkteler (1973) reported the development of haploid embryoids and plantlets from cultured microspores of S. dulcamara L., the European bitter-sweet. However, only degenerating microspores were observed by Zenkteler and Misiura (1974a) in cultured anthers of four other species, namely, S. bulbocastanum Dun., S. chacoense Bitt., S. pinnatisectum Dun., and S. vernei Bitt. & Wittm.

Irikura (1975) successfully induced androgenetic haploids from two Solanum hybrids, S. verrucosum Schlecht. X S. chacoense Bitt., and S. verrucosum Schlecht. X S. tuberosum L., and from several species, namely, S. bulbocastanum, S. demissum Lindl., S. fendleri Heurck & Muell., S. hjertingii J. G. Hawkes, S. phureja Juz. & Buk., S. polytricon Rydb., S. stenotomum Juz. & Buk., and S. stoloniferum Schlecht. & Bouché.

Astley and Weatherhead (1976) reported attempts to trigger androgenic development in S. acaule Bitt. and S. sucrense Hawkes. A few callusing S. sucrense anthers were observed by the authors, but they were unsuccessful with S. acaule.

Recently, the formation of pollen callus from cultured anthers of S. integrifolium Poir. was studied by Sharma and Chowdhury (1977). However, the authors were unable to induce organogenesis.

Sterculiaceae

Theobroma cacao L.

Preliminary studies on anther cultures of the cacao tree have been

recently reported by Esan (1978). Anthers, mostly at the quartet stage, were sown on a modified KC medium supplemented with 0.2 ppm K, 5.0 ppm NAA, and 100 ppm Bacto-peptone. Haploid callus was induced within 35 days and roots developed about one month later. The author also stated that the roots were haploid. Shoots have not yet been produced from the rhizogenic calluses.

Theaceae

Thea sinensis L. (Camellia thea Link)

The regeneration of haploid plantlets from anther cultures of tea was reported by Okano and Fuchinoue (1970).

Tiliaceae

Corchorus capsularis L.

The induction of callus formation from cultured jute anthers has been reported by Iyer and Raina (1972).

Violaceae

Viola wittrockiana Hort. ex Kappert

Unsuccessful attempts to obtain haploid plants or calluses of V. wittrockiana via anther culture were reported by Řerábek and Novotná (1973). None of the culture media used (KC, White (1943), and LS) supplemented with various hormones and organic addenda induced the binucleate pollen grains to divide.

Vitaceae

Vitis species

Gresshoff and Doy (1974) were the first to report the successful culture of haploid cell lines derived from anther cultures of wine grape (*V. vinifera* L.), using a medium designed for tomato anther culture (Gresshoff and Doy, 1972a). They emphasized the importance of the stage of microspore development (quartet to uninucleate) at the time of anther excision and the effect of illumination at the beginning of the culture. Unfortunately, all attempts to differentiate shoots, roots, or plantlets, failed.

Differentiation of shoots and roots from anther-derived callus in a different species, *V. thunbergii* Sieb. et Zucc., was later reported by Hirabayashi *et al.* (1976). The ploidy of these organs was not determined.

The formation of several types of callus tissue from cultured anthers of grapes has also been reported by Tsou *et al.* (1978).

B. Ovary and Ovule Culture

The in vitro culture of female gametophytes as a means of producing haploid plants has received little attention as clearly demonstrated by the paucity of reports available in the literature.

Gymnospermae

A survey of the literature revealed that, in gymnosperms, haploid tissues could easily be derived from the megagametophytes which are massive multicellular structures, in comparison with the female gametophyte of angiosperms.

LaRue (1948, 1955) was the first investigator to report the differentiation of shoots and roots from in vitro-cultured megagametophytes of a cycad, Zamia integrifolia Ait. (Zamia floridana A. DC.). An intermediate callusing phase did not constitute a prerequisite for organogenesis. The ploidy of the regenerated organs could not be determined, but they were presumed to be haploid. Norstog (1965) and Norstog and Rhamstine (1967) later obtained haploid roots and shoots from cultured female gametophytes of this species. These organs were either derived from callus tissues developed from the explant or directly from the explant.

Buds and roots formed on cultured Cycas revoluta Thunb. ovules (LaRue, 1955); these structures were presumed to be haploid. The author also reported limited callus growth from Thuya occidentalis L. megagametophytes and other gymnosperms.

Haploid tissues from Ginkgo biloba L. female gametophytes were recovered by Tulecke (1964, 1967), but he was unable to induce organogenesis.

Similar results were obtained by Norstog and Rhamstine (1967) from cultures of Cycas circinalis L.

More recently, spontaneous polyploidization was observed in haploid calluses derived from cultured megagametophytes of the sugar pine, Pinus lambertiana Dougl. (Borchert, 1968).

Haploid callus cultures have been established from several other members of the Pinaceae, namely, the European larch, Larix decidua Mill. (Bonga, 1976, cited in Nitzsche and Wenzel, 1977), the Japanese larch, L. leptolepis God. (Bonga, 1976, cited in Nitzsche and Wenzel, 1977), the Norway spruce, Picea abies Karst. (Bonga, 1976, cited in Nitzsche and Wenzel, 1977; Huhtinen, 1976), the white spruce, P. glauca Voss (Bonga, 1976, cited in Nitzsche and Wenzel, 1977), the Swiss mountain pine, Pinus mugo Turra (Bonga, 1974, 1976, cited in Nitzsche and Wenzel, 1977), the Austrian pine, P. nigra Arnold (Bonga, 1974, 1976, cited in Nitzsche and Wenzel, 1977), the longleaf pine, P. palustris Mill. (Brown and Sommer, 1975), the red pine, P. resinosa Ait. (Bonga and Fowler, 1970; Bonga, 1976, cited in Nitzsche and Wenzel, 1977), and the pitch pine, P. rigida Mill. (Bonga, 1976, cited in Nitzsche and Wenzel, 1977).

Significant differences were found between six genotypes of Picea abies in their potential in inducing haploid callus from female gametophytes (Huhtinen, 1978).

Angiospermae

Few successful results have been obtained with cultured ovaries and ovules of angiospermous species.

Early reports include those by LaRue (1942) who described rooting of detached ovaries of Asclepias syriaca L. and Armoracia aquatica (Eat.) Wieg. (Radicula aquatica), and profuse callusing from ovules of the snapdragon,

Antirrhinum majus L.; the internal development of the snapdragon ovules did not continue.

Nitsch (1951) did not observed any development from unpollinated Cucumis anguria L. ovaries cultured in vitro. Sachar and Kapoor (1959) reported that unpollinated ovules of a species of Zephyranthes developed into seeds of reduced size one month after the initiation of the culture. Parthenogenetic development of the egg cell or other cells of the embryo sac was not stimulated.

Unfertilized cotton ovules cultured with IAA or GA increased in size and produced fibers (Beasley, 1973). The development of the gametophyte during incubation was not investigated.

In vitro culture of unpollinated ovaries of Vincetoxicum nigrum Moench and Nicotiana tabacum L. was unsuccessful (Haccius, 1974).

Diploid and tetraploid rice plants (Oryza sativa L.) were regenerated from calluses derived from cultured unpollinated ovaries (Nishi and Mitsuoka, 1969).

Haploid cells were detected in calluses obtained from unpollinated ovaries of maize, Zea mays L., which had been pretreated with GA and cultured on various media (Uchimiya et al., 1971). The authors also reported the presence of haploid cells in calluses derived from cultured ovaries of the eggplant, Solanum melongena L.

Successful regeneration of haploid plants via ovary culture has been reported by San Noeum (1976) in barley, H. vulgare L.; cytological examination of the root tips showed the haploid number of chromosomes (7). The recovered plants were all chlorophyllous; this led the author to emphasize the importance of this technique in comparison with anther culture which produces variable numbers of albinos.

C. Inflorescence Culture

The in vitro culture of single flowers has been attempted by several investigators for various purposes (LaRue, 1942; Johri and Guha, 1963; Nitsch, 1963), but whole inflorescences consisting of several buds or spikelets have rarely been cultured.

Miszke and Skucińska (1975) cultured inflorescences of cabbage, Brassica oleracea L., in the course of their investigations on improved techniques of vegetative propagation. Callus tissue often developed from the peduncle and roots were produced from the flower receptacle.

Haploid androgenic plants have been obtained from entire spikes cultured in liquid media (Wilson, 1977; Wilson et al., 1978b). The frequency of pollen callus induction was greatly stimulated by this procedure.

Preil et al. (1978) have recently reported the recovery of haploid plants from cultured heads of Gerbera jamesonii Bolus. Calluses, which developed mainly from the anthers in the tubular florets, subsequently gave rise to haploids.

D. Polyembryony

As illustrated by the phenomenon of polyembryony, haploid plants may arise spontaneously. Polyembryonic seeds may contain two, or more rarely three, seedlings each with its own genotype. Since one member of twin seedlings may be haploid, screening for twins constitutes a valuable tool for the recovery of haploid plants.

Polyembryony, from both the embryological and the cytological points of view, has been the subject of several reviews (Webber, 1940; Kappert, 1950; Maheshwari, 1950; Kimber and Riley, 1963; Magoon and Khanna, 1963; Chase, 1969; Borisenko et al., 1970; Nezhevenko and Shumny,

1970; Lacadena, 1974). Recently, Nitzsche and Wenzel (1977) summarized the data on polyembryonic seeds or twins and found that maternal haploid plants have arisen from these in 64 plant families. Lacadena (1974) listed 41 angiosperm species, belonging to 13 families, in which polyembryony has been reported in connection with haploidy. In the Poaceae, an economically very important family, haploids originating from polyembryonic seeds have been found in several crop species, such as, barley (Müntzing, 1938; Aase, 1946), maize (Chase, 1948, 1963; Rhoades, 1948; Morgan and Rappleye, 1951; Gerrish, 1956; Sarkar and Coe, 1966; Yudin and Khvatova, 1966), rice (Ramiah, 1935; Ramiah et al., 1933, 1938; Choi and Chung, 1961), rye (Müntzing, 1937, 1938; Kostoff, 1939; Levan, 1942), triticale (Straub, cited in Nitzsche and Wenzel, 1977),and wheat (Namikawa and Kawakami, 1934; Yamamoto, 1936; Kasparayan, 1938; Müntzing, 1938; Aase, 1946; Wilson and Ross, 1961; Kihara and Tsunewaki, 1963; Kazaryan et al., 1971; Alamo, cited in Lacadena, 1974).

In legumes, 2n-2n twin seedlings have been reported in red clover, Trifolium pratense L., alsike clover, T. hybridum L., and alfalfa, Medicago sativa L. (Skovsted, 1939); n-2n or n-n twins have only been observed in four species, namely, yellow lupine, Lupinus luteus L. (Kazimierska and Kazimierski, 1970), sweet clover, Melilotus sp. (Jaranowski, 1961),kidney bean, Phaseolus vulgaris L. (Belikova, 1952), Sesbania aculeata Poir. (Haque, 1946) and soybean, Glycine max (L.) Merr. (Kenworthy et al., 1973; Ahmad et al., 1975). The occurrence of twin seedlings has also been reported in peanut, Arachis hypogaea L. (Patel and Natayama, 1935), black medick, Medicago lupulina L., and birdsfoot trefoil, Lotus corniculatus L., but the ploidy of the seedlings was not investigated (Skovsted, 1939).

The mode of origin of the n-n and the n-2n twin seedlings has been studied in a few species and several mechanisms have been proposed.

The occurrence of n-2n twins may be explained by the production of two embryo sacs within the same ovule, one being fertilized while the other develops parthenogenetically, as has been observed in cotton (Harland, 1936; Webber, 1938), rice (Ramiah, 1953), and rye (Müntzing, 1937). Another mode of origin of the n-2n twins may be the result of embryogenesis occurring in synergids or antipodal cells simultaneously with the normal development of the zygote (Kappert, 1933). Conversely, Yamamoto (1936) concluded from his studies of twins in wheat, Triticum aestivum L. (T. vulgare Vill.), that the n-2n twin seedlings origininated from parthenogenetic development of the egg cell and the fertilization of a synergid. Sarkar and Coe (1966) proposed that, in Zea mays, the n-n twins may have resulted from the early cleavage of a single haploid embryo or by simultaneous partheogenetic development of two cells of the embryo sac. Lacadena (1974) suggested that an androgenetic embryo could develop from a sperm nucleus simultaneously with parthenogenetic development of the egg cell or a synergid.

Little is understood of the factors involved in polyembryony. Kappert (1950) concluded from his studies on flax that the frequency of polyembryony was controlled by recessive genes which produced maternal and zygotic effects. Results obtained by various researchers have shown variations in the frequency of polyembryonic seeds from one species to another and sometimes within strains of the same species (Christensen and Bamford, 1943; Morgan and Kappleye, 1950). Campos and Morgan (1960) produced evidence that the frequency of twinning in pepper is controlled by the genotype of the female parent.

The importance of twin selection as one of the technique for recovering haploid plants has recently been stressed (Nitzsche and Wenzel,

1977); the outcome of a screening for twin seedlings in Lotus species may well lead to the recovery of haploid plants.

II

MATERIAL AND METHODS

A. Anther culture

1. Material and Growing Conditions

The various sources of Lotus germplasm used for the anther culture experiments are listed in Table IV.

The plants were grown in pots and kept in the greenhouse from September to May. During the summer months, the pots were taken outside and placed in cold frames. The perennial species were repotted every six months and both perennial and annual species were regularly fertilized with a 20-20-20 (CIL) solution and checked for the presence of pests and disease symptoms.

In the greenhouse, the plants were kept under a 16-hour photoperiod in order to ensure abundant flowering (Grant et al., 1962). Illumination was provided by fluorescent lamps placed 1.5 meters above the benches. Temperatures approximated 21°C during the daytime and 18° at night.

2. Flower Bud Selection and Preparation of the Explants

Anthers at various stages of development, that is from quartets to mature pollen grains, were utilized. Special emphasis was placed on uninucleate microspores as there are a great number of successful reports of haploid plants obtained from anthers cultured at this stage as for instance in barley (Foroughi-Wehr et al., 1976), belladonna (Zenkteler,

TABLE IV. *Lotus* species used for anther culture

Species	Genetics Laboratory accession number	2n	Source
Diploid			
L. glaucus Ait.	B348	14	Jardin Canario "Viera y Clavijo", Gran Canaria, Canary Islands.
Amphidiploid			
(*L. japonicus* (Regel) Larsen X *L. alpinus* Schleich.)^2	A/JA	24	Produced by Dr. B.H. Somaroo (1970), Macdonald College, Ste-Anne de Bellevue (Que.) Canada.
Tetraploids			
L. angustissimus L.	B141	24	Origin, Israel. Division of Plant Industry, C.S.I.R.O., Canberra, Australia. C.P.I. 15606
L. corniculatus L.	B259	24	Botanischen Garten und Museum Berlin-Dahlem, Germany.
L. corniculatus L. cv. Leo	AA764	24	Produced from seeds of cv. Leo (B764) treated with 50 ppm of diiodo-L-tyrosine. G. Séguin (1977), Macdonald College, Ste-Anne de Bellevue (Que.) Canada.
L. corniculatus L. cv. Mirabel	AA779	24	Produced from seeds of cv. Mirabel (B779) treated with 25 ppm of diiodo-L-tyrosine. G. Séguin (1977), Macdonald College, Ste-Anne de Bellevue (Que.) Canada.

Continued...

TABLE IV. (Continued)

Species	Genetics Laboratory accession number	2n	Source
L. corniculatus L. cv. Leo	EMS 764	24	Produced from seeds of cv. Leo (B764) treated with 0.01% EMS. M.C. Therrien (1976), Macdonald College, Ste-Anne de Bellevue (Que.), Canada
L. cruentus Court.	B79	28	Botanic Garden of Adelaide, South Australia.
L. emeroides Murray	B349	28	Jardin Canario "Viera y Clavijo", Gran Canaria, Canary Islands.
L. mascaensis Buchard	B350	28	Jardin Canario "Viera y Clavijo", Gran Canaria, Canary Islands.
L. tenuis Waldst. et Kit.	B340	24	Seed obtained from Dr. A. Gershoy, University of Vermont, Burlington Vt., USA.
L. villosus Forsk.	B189	28	Origin, Israel. Plant Introduction Station, Geneva(N.Y.), USA.
Synthetic autotetraploids			
L. alpinus Schleich.	T77	24	Tetraploid produced from L. alpinus (B77) by Dr. B.H. Somaroo (1970), Macdonald College, Ste-Anne de Bellevue (Que.), Canada.
L. japonicus (Regel) Larsen	T129	24	Tetraploid produced from L. japonicus (B129) by Dr. B.H. Somaroo (1970), Macdonald College, Ste-Anne de Bellevue (Que.), Canada.

1971; Rashid and Street, 1973), eggplant (Anonymous, 1978d), Lycium halimifolium (Zenkteler, 1972), potato (Dunwell and Sunderland, 1973), rice (Guha-Mukherjee, 1973; Chen, 1977) Solanum dulcamara (Zenkteler, 1973), triticale (Bernard, 1977; Dorosiev et al., 1978), and wheat (Craig, 1974). The different stages of anther development could be correlated with the morphological development of the flower buds, which made it possible to easily select a particular pollen developmental stage by choosing the appropriate flower buds. Anthers were staged according to their development, as follows: stage 1, included pollen mother cells to quartets; stage 2, uninucleate microspores; and stage 3, grains at the first pollen mitosis to mature binucleate pollen grains.

Flower buds at the selected stage of development were collected from the donor plants and, in some cases, given a cold pretreatment. This consisted in arranging the buds between two sheets of moistened filter paper in a petri dish and placing the petri dish in the dark at 4°C for a period of 24 hours.

Surface-sterilization of the buds was achieved by dipping each flower bud in 70% ethanol for 10 seconds, followed by three minutes in a 7% solution of calcium hypochlorite, and a five minute rinse in sterile distilled water. The buds were then transferred to a sterile petri dish which was placed in a dissecting hood that had been presterilized with ultraviolet light for 30 minutes. Dissecting tools, glassware, microscopic slides, filter paper sheets, etc. used during dissection of the buds, were oven sterilized at 160°C for two hours.

The filaments were generally detached from the anthers which were then sown on the various culture media (Fig. 1); in some cases,

the ovary was placed with the anthers in the same culture bottle (Fig.2).

3. Culture Medium Formulation and Preparation

Three synthetic culture media, namely, Murashige and Skoog (1962; Appendix 1), Gamborg (1970; Appendix 2), Veliky and Martin (1970; Appendix 3), and undefined medium, termed Potato culture medium (Appendix 4), were utilized in the anther culture studies. The basic culture media were supplemented with a variety of growth regulators and organic substances. Details are given below.

a) Murashige and Skoog (1962)

The basic medium was modified in several ways.

Type I

Firstly, the basic medium was used as formulated and supplemented with 3% sucrose, coconut milk, and 2,4-D (Table V).

Type II

Secondly, the concentration of the minerals, i.e. macroelements, microelements, and iron, were reduced by half. The concentration of the vitamins and the myo-inositol remained unchanged. Various levels of sucrose and two growth regulators were used to supplement the medium (Table VI).

Type III

Thirdly, the concentration of all the componenets of the basic medium, i.e. minerals, vitamins and myo-inositol, were halved. Sucrose (3%) and various growth regulators (Table VII) were added to the medium.

TABLE V. Type I. Coconut milk and 2,4-D concentrations used in combination with Murashige and Skoog's culture medium (1962)

Medium subtype	CM (% v/v)	2,4-D (mg/1)
1	—	—
2	15	—
3	30	—
4	15	1.0

TABLE VI. Type II. Growth regulators and sucrose levels used in combination with Murashige and Skoog's culture medium (1962)

Medium subtype	Growth regulators mg/1		Sucrose (% w/v)
	2,4-D	BN	
1	0.5	—	8
2	0.5	—	12
3	—	2.0	3
4	0.1	2.0	3

TABLE VII. Type III. Growth regulators used in combination with Murashige and Skoog's culture medium (1962)

Medium subtype	Growth regulators (mg/l)		
	2,4-D	IAA	BAP
1	---	---	1.0
2	---	---	2.0
3	1.0	---	2.0
4	2.0	---	1.0
5	---	2.0	1.0
6	---	1.0	2.0

b) Gamborg (1970)

The basic medium was used as formulated and supplemented with 3% sucrose and several growth regulators (Table VIII).

TABLE VIII, Growth regulators used in combination with Gamborg's culture medium (1970)

Medium subtype	Growth regulators (mg/l)				
	2,4-D	NAA	α,β-D	K	BAP
1	---	---	---	---	---
2	---	---	1.0	---	---
3	---	1.0	---	0.1	---
4	1.0	---	---	0.1	---
5	1.0	---	1.0	0.1	---
6	---	---	---	---	2.0

continued...

TABLE VIII. (Continued)

Medium subtype	Growth regulators (mg/l)				
	2,4-D	NAA	α, β-D-	K	BAP
7	0.5	---	---	---	1.0
8	---	0.5	---	---	1.0

c) Veliky and Martin (1970)

The basic medium was utilized as formulated and supplemented with 2% sucrose and various growth regulators (Table IX).

TABLE IX. Growth regulators used in combination with Veliky and Martin's culture medium (1970)

Medium subtype	Growth regulators (mg/l)			
	2,4-D	IAA	NAA	K
1	---	---	---	---
2	1	---	---	---
3	---	2	1	0.2
4	---	---	0.5	---

The components of the three synthetic culture media were prepared in a similar way.

Double (glass) distilled water was used in the preparation for each component in each of the different culture media. Stock solutions for macroelements, microelements, and iron were kept refrigerated and replaced after two months. Vitamins and myo-inositol stock solutions

were stored in a freezer. Stock solutions (0.5 mg/ml) of growth regulators were prepared immediately before use in the following manner.

Auxins (IAA, NAA, α,β-D, and 2,4-D)

For each auxin, 50 mg were first dissolved in a minimal volume of 95% ethanol; the solution was gently heated and double distilled water was added very slowly, up to a volume of 100 ml.

Cytokinins (K, BAP, and Z) and BN

For each compound, 50 mg were initially dissolved in a small amount of 0.1 N NaOH or 0.1 N HCl and the solution was gradually made up to 100 ml with double distilled water.

Coconut milk was prepared as follows. Ripe coconuts were purchased from a local grocery store. A hole was drilled through one of the germination pores and the liquid was collected, heated to 80°C (with stirring), and filtered through several layers of cheese cloth. The filtrate was then set aside to cool to room temperature and stored frozen.

The culture media were prepared by combining predetermined volumes of the stock solutions (macroelements, microelements, iron, vitamins, myo-inositol, and growth regulators), dissolving the sucrose, and making the solution up to the required volume with double distilled water. The pH was then adjusted to the required level (5.7-5.8 for Murashige and Skoog's medium, 6.0 for Gamborg's medium, and 4.5 for Veliky and Martin's medium) with a small amount of 0.1 N HCl or 0.1 N NaOH.

Liquid media were poured into 30 cc clear glass bottles

(Ampak Ltd.), rinsed with double distilled water and labelled with colored tape (Professional Tape Co.) for coding purposes. The bottles contained a cotton ball topped with a filter paper disk (Fig. 3) in order to support the explants. About 20 ml of media were necessary to completely wet the cotton ball and the piece of filter paper.

In order to prevent leaching of chemicals from the cotton wool and the filter paper, these were cleaned by rinsing several times in double distilled water, followed by autoclaving at 121°C for 15 to 20 minutes, and rinsing again several times with double distilled water. The cotton balls and the filter paper disks were then dried in the oven at 60°C overnight.

Contamination of the media by chemicals present in new bottles and caps was prevented by soaking the bottles in a chromic acid solution for 24 hours, followed by rinsing for 48 hours in tap water; the plastic caps (Ampak Ltd.) were soaked in a 1 N HCl solution for 24 hours and rinsed in tap water for 24 to 36 hours.

For solid culture media, Bacto-agar (0.6%) was used as the gelling agent. The agar was slowly dissolved by heating and stirring. In some cases, activated charcoal (0.5%) was added to the culture media. Ten milliliters of media were poured into each culture bottle, prerinsed with double distilled water and labelled with colored tape. The caps were screwed on tightly and the bottles were autoclaved at 121°C for 20 minutes.

d) Potato Culture Medium

The medium used was similar to the one described by a Chinese research group for wheat anther culture (Anonymous, 1976). It

consisted of the following ingredients: Potato extract 20%, FeNaEDTA 40 mg/l, and sucrose 9%. Various growth regulators were used in combination with this medium (Table X).

TABLE X. Growth regulators used in combination with the Potato Culture Medium

Medium subtype	Growth regulators (mg/l)		
	2,4-D	BN	K
1	—	—	—
2	2.0	—	0.5
3	2.0	1.0	0.5
4	2.0	—	—

The Potato Culture Medium was prepared in the following manner. The potato tubers (cv. Kennebec) were washed with tap water and in one instance, peeled; the buds were removed and the tubers were diced; 200 grams of potato cubes were then boiled in 500 ml of double distilled water for 30 minutes. A Waring blender was then used to liquefy the potato mixture, and the resulting purée was filtered through several layers of cheese cloth. Sucrose, FeNaEDTA, and in the case of medium subtypes 2,3 and 4 (Table X), growth regulators, were added to the filtrate. The solution was made up to one liter with double distilled water and the pH was adjusted to 5.7 to 5.8. In some cases, activated charcoal (0.5%) was added to the medium. Bacto-agar (0.6%) was used to gel the medium, as previously described.

Culture bottles and plastic caps identical to those used for the

synthetic culture media were utilized. Each bottle was labelled with a piece of colored tape and rinsed with double distilled water prior to use. Ten milliliters of potato medium were poured into each culture bottle. The caps were adjusted and the medium was autoclaved at 121°C for 20 minutes.

4. Cultural Conditions

The cultures were incubated in growth chambers at different temperatures and were submitted to various photoperiods (Table XI).

TABLE XI. Photoperiods and temperatures used for anther culture

Treatment	Duration of regime	Photoperiod		Temperature	
		day (h)	night (h)	day (°C)	night (°C)
a	Continuous	24	0	25	—
b	Continuous	24	0	30	—
c	10 days, then	0	24	—	30
	continuous	24	0	25	—
d	10 days, then	0	24	—	25
	continuous	24	0	25	—
e	10 days, then	24	0	30	—
	continuous	24	0	25	—

Incubation was performed under low light intensity (16 $\mu Esec^{-1}$ m^{-2}, ca. 800 lux), and illumination was provided by fluorescent lamps (Cool-White, General Electric, or Vita Lite, Duro-Test Electric Ltd.). Relative humidity was maintained at the 60 to 65% level.

5. Callus Subculture Procedures

Callused anthers were transferred to a differentiation medium in order to induce regeneration of plantlets. This culture medium consisted of Murashige and Skoog's basal medium supplemented with 1 ppm K, 0.1 ppm NAA, and 3% sucrose. The medium was gelled with 0.6% Bacto-agar and a volume of ten milliliters was poured into each culture bottle which was then capped and autoclaved, as described in Section 3.

Calluses were incubated at 25°C in continuous light and regularly subcultured on the regeneration medium until plantlets were developed. It was found unnecessary to use a basal medium, that is, unsupplemented with growth regulators, to induce rooting.

6. Plantlet Transfer

Plantlets were delicately removed from the culture vessels and placed in 10-cm plastic pots filled with a light 1:1:1 mixture of peat moss, perlite and vermiculite. No attempts were made to remove the agar covering the root system, so that damage to the fragile roots would be prevented. One-liter beakers were placed upside down over the pots to create a highly humid environment which reduced dehydration of the plantlets. The covered pots were then transferred to the greenhouse. The beakers were removed after 10 days and the plantlets were later repotted in a regular soil mixture and grown to maturity.

7. Cytological Examination of Cultured Anthers, Callus Cells, and Regenerants

a) Cultured Anthers

In general, a few anthers from each treatment were examined after one month in culture, especially when callus tissue had not been induced

to form; callused anthers were usually subcultured, as described in Section 5. The anthers were gently teased with dissecting needles, then squashed in a drop of aceto-carmine and examined a few hours later under a microscope.

b) Callus Cells

Small portions of tissue were removed aseptically from the surface of the callus mass and pretreated in 0.002 M 8-hydroxyquinoline for two hours at room temperature. The pieces of callus were then rinsed with distilled water and fixed in Carnoy's fluid (three parts of 95% ethanol to one part of glacial acetic acid) for 12 to 16 hours at 4°C. Following several rinses with distilled water, the fixed pieces of callus were macerated in a 4% pectinase solution for 6 to 12 hours at room temperature. The tissues were then rinsed with distilled water and stained with an alcoholic hydrochloric acid-carmine solution (Snow, 1963) for about 12 hours at 60°C. Slides were prepared according to a routine squashing technique.

c) Regenerants

The determination of the chromosome number was performed by examining mitotic (root tips) or meiotic (pollen mother cells) tissues.

i) Root tips

Healthy root tips were collected from the regenerants and pretreated in 0.002 M 8-hydroxyquinoline for two hours at room temperature. Distilled water was used to rinse the root tips which were then fixed in Carnoy's fluid for 12 to 16 hours at 4°C. The fixed root tips were then rinsed several times with distilled, and once with 1 N HCl at room temperature. Hydrolysis was achieved by adding hot

1 N HCl (60°C) to the vials and placing them in a water bath at 60°C for 6 to 8 minutes. The vials were then transferred to an ice water bath to stop the reaction, and the root tips were rinsed several times with distilled water. Staining was performed at 4°C according to a standard Feulgen procedure (Darlington and La Cour, 1960). The root tips were again rinsed with distilled water and macerated in a 4% pectinase solution for 3 to 5 hours. The root tips were finally rinsed with distilled water, transferred to 70% ethanol, and stored at 4°C. Preparation of the slides was done according to a routine squashing method.

ii) Pollen Mother Cells

Young flower buds were collected from the mature regenerated plants and fixed in a modified Carnoy's solution (six parts of absolute ethanol to three parts of chloroform to one part of glacial acetic acid) for two hours at 4°C. The buds were then rinsed with distilled water, and stained with alcoholic hydrochloric acid-carmine for about 12 hours at 60°C. The buds were either left in the stain or transferred to 70% ethanol, and stored at 4°C. A routine squashing technique was used for preparing the slides.

Pollen viability of regenerated plants was estimated according to the procedure outlined by Marks (1954). A mature undehisced anther was macerated in a small drop of aceto-carmine jelly which gives an even distribution of both empty and plump pollen grains. Debris was removed and the coverslip was applied. The preparation was examined under a microscope a few hours later when staining and differentiation of the pollen grains was complete. About 1000 empty and full pollen grains were counted from one edge of the coverslip to the other to

obtain representative results. Shrivelled and unstained pollen grains were scored as inviable; conversely, plump, normal-sized pollen grains which took up the stain were counted as viable.

8. Statistical Analysis

The proportion of callusing anthers on various media was analyzed using the comparison of proportions for two binomial populations (Ostle and Mensing,1975). The chi-square approximation, corrected for continuity, to the binomial distribution was used to test the null hypothesis of no medium or within medium condition differences. All possible permutations of the media with identical conditions and all conditions within a medium were tested in pairs using the above statistical analysis.

B. Pollen Culture

1. Material and Growing Conditions

Two tetraploid Lotus species were used in these studies (Table XII). The donor plants were grown under greenhouse conditions from September to May and kept outdoors in cold frames during the summer months. Details on the growing conditions are given in Section A-1.

TABLE XII. *Lotus* germplasm used for pollen culture

Species	Genetics Laboratory accession number	2n	Source
L. corniculatus L.	B534	24	Origin, Yugoslavia. Seed obtained by Dr. J. Rubar through the University of Yugoslavia.
L. emeroides Murray	B349	28	Jardin Canario "Viera y Clavijo", Gran Canaria, Canary Islands.

2. Preparation of the Pollen Suspensions, Culture Medium Formulation and Preparation, and Culture Procedures

The flower buds were collected from the donor plants and surface-sterilized with 70% ethanol for ten seconds, followed by three minutes in an agitated solution of 7% calcium hypochlorite. The buds were then thoroughly rinsed with sterile distilled water and transferred to a sterile petri dish. Dissection of the flower buds was done aseptically in a dissecting hood.

The culture of isolated microspores was performed in several ways.

Firstly, young anthers containing pollen mother cells up to the quartet stage (Stage 1) were homogenized in a few milliliters of liquid culture medium to form a suspension. Two to three drops of the suspension were placed in well slides in sterile petri dishes and each well slide was covered with a sterile microscopic slide to prevent evaporation. The culture medium consisted of the basic medium

of Murashige and Skoog (1962; Appendix 1), supplemented with 1 ppm IAA, 0.1 ppm K, and 3% sucrose. The cultures were incubated in the dark at 25°C.

Secondly, anthers at the uninucleate stage (Stage 2) were gently homogenized with a glass rod in three milliliters of sterile distilled water; a few drops of the microspore suspension were then spread on the surface of a solid culture medium. This medium consisted of the basic medium of Gamborg (1970; Appendix 2), supplemented with 3% sucrose and gelled with Bacto-agar (0.6%). The cultures were incubated in the dark at 25°C.

Thirdly, mature, dehiscing anthers (Stage 3) were sown on a Gamborg's B5 (1968)-soybean medium (Appendix 5), supplemented with 5 ppm 2,4-D and gelled with Bacto-agar (0.6%). The culture medium was prepared as follows. Soybean seeds (100 g) were washed with tap water and soaked in distilled water for 21 to 14 hours. The imbibed seeds were then drained, rinsed with double distilled water, and ground in approximately 300 milliliters of double distilled water. The resulting thick liquid was then filtered through several layers of cheese cloth and the filtrate was gently brought to a boil (with stirring). Required volumes of stock solutions of Gamborg's basic medium (macroelements, microelements, and iron) and 2,4-D were added to the soybean extract. Sucrose (10%) was dissolved and the solution was made up to one liter with double distilled water. The pH was estimated with indicator paper as approximately 6.

The agar was slowly dissolved by heating and the medium was autoclaved in 250-ml erlenmeyer flasks sealed with aluminum foil for 20 minutes at 121°C. The sterilized medium was then poured aseptically in sterile 60 X 15-mm plastic petri dishes. The cultures

were incubated in the dark at two different temperatures, 25°C and 30°C.

Details pertaining to the preparation of the stock solutions of the different culture media, the growth regulators, and the complete culture media, as well as the culture vessels and sterilization procedures, are given in Section A-3.

3. Examination of the Cultures

The cultures were examined after two to five weeks of incubation for the presence of callus tissue or pollen embryoids.

In the case of microspores sown on solid culture media, a small amount of material was removed aseptically from the surface of the agar with a bacteriological loop, placed on a microscopic slide and stained with aceto-carmine. The coverslip was applied and the mount was firmly pressed down.

The development of microspores inoculated in liquid medium was investigated by removing the slide covering the well slide, adding one small drop of aceto-carmine to the suspension in the well, and examining the slide a few minutes later under a microscope.

C. Pollen Tube Culture

1. Material and Growing Conditions

One amphidiploid and five tetraploid Lotus species were used in these studies (Table XIII).

The donor plants were grown either in the greenhouse or outdoors in cold frames, under the growth conditions described in Section A-1.

TABLE XIII. *Lotus* germplasm sources used for the *in vitro* culture of pollen tubes

Species	Genetics Laboratory accession number	2n	Source
L. corniculatus L.	B259	24	Botanischen Garten und Museum Berlin-Dahlem, Germany.
L. cruentus Court.	B79	28	Botanic Garden of Adelaide, South Australia.
L. mascaensis Buchard	B350	28	Jardin Canario "Viera y Clavijo", Gran Canaria, Canary Islands.
L. pedunculatus Cav.	B124	24	Tetraploid produced by P.I. Barclay, Department of Scientific and Industrial Research, Grasslands Division, Palmerston North, New Zealand.
(*L. krylovii* Schiachk. et Serg. X *L. tenius* Waldst. et Kit.)[2]	A/KT	24	Amphidiploid produced by Dr. B.H. Somaroo (1970), Macdonald College, Ste-Anne de Bellevue (Que.), Canada.

2. Sterilization Procedures and Germination of the Pollen

Mature flowers were collected from the donor plants and surface-sterilized with 70% ethanol for ten seconds, followed by three minutes in an agitated solution of 7% calcium hypochlorite; the florets were then thoroughly rinsed with sterile distilled water and transferred to a sterile petri dish.

Dissection of the florets was performed aseptically in a

laminar air flow cabinet; the mature pollen grains were removed from the keel with a dissecting needle and placed into 10-ml erlenmeyer flasks containing five milliliters of the germination medium. The medium consisted of a 0.3 M solution of sucrose, supplemented with 0.001% boric acid. This solution was filter-sterilized (Millipore Corporation) and aseptically poured into flasks which were presterilized in an oven at 160°C for two hours. Aluminium foil was used to seal the flasks.

The flasks were incubated in the dark at 25°C until a sample showed a large proportion of germinating pollen grains, generally occurring after five to eight days of incubation.

3. Culture Medium Formulation and Preparation

Murashige and Skoog's culture medium (1962; Appendix 1) was utilized in these studies. The basic medium was used as formulated by the authors and supplemented with 3% sucrose and 2 ppm 2,4-D. The culture medium was gelled with Bacto-agar (0.6%) and poured into glass bottles.

Details concerning the preparation of the various constituents of the Murashige and Skoog's medium 2,4-D, and complete culture medium, as well as the description of the culture vessels and the sterilization procedures can be found in Section A-3.

4. Cultural Conditions

Three to four drops of the pollen grain suspension were spread onto the surface of the culture medium. The cultures were incubated in the dark at 25°C.

5. Examination of the Cultures

The cultures were examined after one month of incubation to determine whether divisions in the pollen tubes and callus tissue formation had been induced.

Material on the surface of the medium was gently removed with a bacteriological loop, spread on a microscopic slide and stained with aceto-carmine. A coverglass was then placed into position and the mount was firmly pressed down.

D. Ovary and Ovule Culture

1. Material and Growing Conditions

The different Lotus species utilized for the in vitro culture of ovaries and ovules are listed in Table XIV.

The donor plants were grown under greenhouse conditions from September to May and kept outdoors in cold frames during the summer months. Details pertaining to the growing conditions are given in Section A-1.

2. Flower Bud Selection and Preparation of the Explants

The culture of ovaries and ovules was usually performed concurrently with anther culture. The ovaries were staged similarly to the anthers: stage 1 (pollen mother cells to quartets in the anthers), ovaries containing ovule primordia which were beginning to elongate and bend; stage 2 (early to late uninucleate microspores), ovaries containing premeiotic ovules to ovules with very young embryo sacs: and stage 3 (early binucleate to mature pollen grains), ovaries containing ovules with developing to fully mature embryo sacs.

The flower buds at the selected stage of development were

TABLE XIV. Lotus species used for ovary and ovule culture

Species	Genetics Laboratory accession Number	2n	Source
Diploids			
L. glaucus Ait.	B348	14	Jardin Canario "Viera y Clavijo", Gran Canaria, Canary Islands.
L. krylovii Schischk. et Serg.	B86	12	Hortus Botanicus Universitatis, Uppsala, Sweden
Amphidiploid and hybrids			
(L. japonicus (Regel) Larsen X L. alpinus Schleich.)^2	A/JA	24	Produced by Dr. B.H. Somaroo (1970), Macdonald College, Ste-Anne de Bellevue (Que.), Canada.
(L. japonicus (Regel) Larsen X L. alpinus Schleich.) X L. corniculatus L.	JA-2-15 X 554	24	Produced by Dr. B.H. Somaroo (1970), Macdonald College, Ste-Anne de Bellevue, (Que.), Canada.
(L. krylovii Schischk. et Serg. X 554$_2$ X L. schoelleri Schweinf.)^2 X L. corniculatus L.	KS 7(a)5	24	Produced by Dr. B.H. Somaroo (1970), Macdonald College, (Que.), Canada
Tetraploids			
L. angustissimus L.	B141	24	Origin, Israel. Division of Plant Industry, C.S.I.R.O., Canberra, Australia. C.P.I. 15606
L. corniculatus L.	B259	24	Botanischen Garten und Museum Berlin-Dahlem, Germany

Continued...

TABLE XIV. (Continued)

Species	Genetics Laboratory accession number	2n	Source
L. corniculatus L.	B280	24	Origin, Poland. Plant Introduction Station, Geneva (N.Y.) USA. P.I. 255176
L. corniculatus L.	B285	24	Origin, Korea. Plant Introduction Station, Geneva (N.Y.) USA. P.I. 273443
L. corniculatus L.	B534	24	Origin, Yugoslavia. Seed obtained by Dr. J. Bubar through the University of Yugoslavia.
L. corniculatus L.	B554	24	Origin, Turkey. Plant Introduction Station, Izmir, Turkey.
L. corniculatus L. cv. Mirabel	B779	24	Commercial certified seed. Plant Science Department, Macdonald College, Ste-Anne de Bellevue (Que.), Canada.
L. corniculatus L. cv. Leo	AA764	24	Produced from seeds of cv. Leo (B764) treated with 50 ppm diiodo-L-tyrosine. G. Séguin (1977), Macdonald College, Ste-Anne de Bellevue (Que.), Canada.
L. corniculatus L. cv. Leo	EMS764	24	Produced from seeds of cv. Leo (B764) treated with 0.01% EMS. M.C. Therrien (1976), Macdonald College, Ste-Anne de Bellevue (Que.), Canada.

Continued...

TABLE XIV. (Continued)

Species	Genetics Laboratory accession number	2n	Source
L. cruentus Court.	B79	28	Botanic Garden of Adelaide, South Australia.
L. emeroides Murray	B349	28	Jardin Canario "Viera y Clavijo", Gran Canaria, Canary Islands.
L. mascaensis Buchard	B350	28	Jardin Canario "Viera y Clavijo", Gran Canaria, Canary Islands.
L. tenuis Waldst. et Kit.	B340	24	Seed obtained from Dr. A. Gershoy, University of Vermont, Burlington (Vt.), USA.
L. villosus Forsk.	B189	28	Origin, Israel. Plant Introduction Station, Geneva (N.Y.), USA. P.I. 238336
Synthetic autotetraploids			
L. alpinus Schleich.	T77	24	Produced from L. alpinus (B77) by Dr. B.H. Somaroo (1970), Macdonald College, Ste-Anne de Bellevue (Que.), Canada.
L. japonicus (Regel) Larsen	T129	24	Produced from L. japonicus (B129) by Dr. B.H. Somaroo (1970), Macdonald College, Ste-Anne de Bellevue (Que.), Canada.

harvested from the donor plants and, in some cases, given a cold treatment which is detailed in Section A-2.

The flower buds were surface-sterilized as previously described in Section A-2. The ovaries with or without anthers were placed on the surface of the culture medium (Fig. 2), or planted in the agar (Fig. 4).

3. Culture Medium Formulation and Preparation

The ovaries and the ovules were cultured on the media used for anther culture, namely, Murashige and Skoog (1962; Appendix 1), Gamborg(1970; Appendix 2), Veliky and Martin (1970; Appendix 3), and the Potato medium (Appendix 4). The basal media were supplemented with various growth regulators and organic substances, as detailed below.

a) Murashige and Skoog (1962)

The same modifications were brought to the Murashige and Skoog's medium (Type I, II, and III), as described in Section A-3-a. These media were supplemented with various growth regulators and organic addenda (Tables XV, XVI). Type II medium was supplemented with 0.5 ppm 2,4-D and 8% or 12% sucrose.

TABLE XV. Type I. Growth regulators and organic supplement used in combination with Murashige and Skoog's culture medium (1962)

Medium subtype	Growth regulators (mg/l)			Org. suppl. (% v/v)
	2,4-D	IAA	Z	CM
1	—	—	—	—
2	—	0.1	—	—

Continued...

TABLE XV. (Continued)

Medium subtype	Growth regulators (mg/l)			Org. suppl. (% v/v)
	2,4-D	IAA	Z	CM
3	---	---	0.1	---
4	---	0.1	0.1	---
5	---	---	---	15
6	---	---	---	30
7	1.0	---	---	15

TABLE XVI. Type III. Growth regulators used in combination with Murashige and Skoog's culture medium (1962)

Medium subtype	Growth regulators (mg/l)		
	2,4-D	IAA	BAP
1	1.0	---	---
2	---	---	1.0
3	---	---	2.0
4	1.0	---	2.0
5	2.0	---	1.0
6	---	2.0	1.0
7	---	1.0	2.0

b) Gamborg (1970)

The basic medium was utilized as formulated and supplemented with different growth regulators and 3% sucrose (Table XVII).

TABLE XVII. Growth regulators used in combination with Gamborg's culture medium (1970)

Medium subtype	Growth regulators (mg/l)					
	2,4-D	NAA	NlAA	α,β-D	K	BAP
1	---	---	---	---	---	---
2	1.0	---	---	---	---	---
3	---	---	---	1.0	---	---
4	---	1.0	---	---	0.1	---
5	---	0.1	---	---	1.0	---
6	1.0	---	---	---	0.1	---
7	1.0	---	---	1.0	0.1	---
8	---	---	---	---	---	2.0
9	0.5	---	---	---	---	1.0
10	---	0.5	---	---	---	1.0
11	---	---	0.5	---	---	1.0

c) Veliky and Martin (1970)

The medium was utilized as formulated and supplemented with 2% sucrose. The explants were cultured on subtypes 3 and 4 (see Table IX).

d) Potato Culture Medium

The medium was prepared as described in Section A-3-d and supplemented with the growth regulators listed in Table X.

Details on the preparation of the synthetic media, the Potato medium the coconut milk, and the growth regulators, and the description of the culture vessels and the sterilization procedures are given in Section A-3.

4. Cultural Conditions

The cultures were incubated in growth chambers at different temperatures and under various light regimes (Table XVIII). Incubation was performed under low light intensity (16 $\mu E\ sec^{-1}\ m^{-2}$, ca. 800 lux), and illumination was provided by fluorescent lamps (Cool-White, General Electric, or Vita-Lite, Duro-Test Electric Ltd.). Relative humidity was maintained at the 60 to 65% level.

TABLE XVIII. Light regimes and temperatures used for ovary and ovule culture

Treatment	Duration of regime	Photoperiod day (h)	Photoperiod night (h)	Temperature day (°C)	Temperature night (°C)
a	Continuous	16	8	25	25
b	Continuous	24	0	25	--
c	Continuous	24	0	30	--
d	10 days, then	0	24	--	30
	continuous	24	0	25	--
e	10 days, then	0	24	--	25
	continuous	24	0	25	--
f	10 days, then	0	24	--	30
	continuous	24	0	30	--

5. Callus Subculture Procedures

The subculture of calluses was performed as described for calluses obtained from anthers. Details are given in Section A-5.

6. Plantlet Transfer

The method described in Section A-6 for transferring plantlets was utilized.

7. Cytological Studies

In general, non-callusing ovaries were dissected after one to two months of incubation to examine the ovules. Callused ovaries and ovules were subcultured, as previously described (Section A-5). The fixation and the staining procedures used in the study of the callus cells are outlined in Section A-7-b.

8. Statistical Analysis

The proportion of callusing ovaries on various culture media was analyzed using the comparison of proportions for two binomial populations, as described in Section A-8.

E. In vitro Culture of Inflorescences

1. Material and Growth Conditions

The Lotus germplasm sources used for the culture of inflorescences are listed in Table XIX.

The plants were grown under the same conditions as described in Section A-1.

TABLE XIX. *Lotus* germplasm used for inflorescence culture

Species	Genetics Laboratory accession number	2n	Source
L. alpinus Schleich. (4x)	T77	24	Tetraploid produced by Dr. B.H. Somaroo (1970), Macdonald College, Ste- Anne de Bellevue (Que.), Canada.
L. angustissimus L.	B141	24	Origin, Israel. Division of Plant Industry, C.S.I.R.O., Canberra, Australia.
(*L. burtii* Sz.-Borsos X *L. filicaulis* Dur.)^2 X 554 X *L. corniculatus* L.	303-F-2-3	24	Hybrid produced by Dr. B. H. Somaroo (1970), Macdonald College, Ste-Anné de Bellevue (Que.), Canada
(*L. japonicus* (Regel) Larsen X *L. alpinus* Schleich.)^2 X *L. corniculatus* L.	JA-10 X 554	24	Hybrid produced by Dr. B. H. Somaroo (1970), Macdonald College, Ste-Anne de Bellevue (Que.), Canada.

2. Preparation of Explants

Inflorescences bearing three to six flower buds at various stages of development were collected from the donor plants. The bractlets and, in the case of very young inflorescences, the leaves surrounding the flowering apex were removed.

Surface-sterilization was achieved by dipping the inflorescences in 70% ethanol for ten seconds, then transferring them to an agitated solution of 7% calcium hypochlorite to which a drop of a wetting agent, Tween 20, had been added. After ten minutes, the inflorescences were

rinsed thoroughly with sterile distilled water and transferred to a sterile petri dish.

3. Culture Medium Formulation and Preparation

A modified Murashige and Skoog's culture medium (1962; Appendix 1) was used in these studies. The concentration of the mineral constituents (macroelements, microelements, and iron) was reduced by half; the concentration of the vitamins and the myo-inositol remained unchanged. The basal medium was supplemented with 3% sucrose and two growth regulators, namely, 2,4-D and benazolin (Table XX).

TABLE XX. Growth regulators used in combination with modified Murashige and Skoog's culture medium

Medium subtype	Growth regulators (mg/l)	
	2,4-D	BN
1	—	2
2	—	4
3	0.1	2

Solid and liquid culture media were utilized. Bacto-agar (0.6%) was used as the gelling agent. In some cases, activated charcoal (0.5%) was added to the solid culture media. Liquid media were poured into bottles in which a cotton ball had been placed in order to support the explant.

Details concerning the preparation of the various constituents

of the Murashige and Skoog's medium, 2,4-D, benazolin, and the complete culture media, as well as the description of the culture vessels and the sterilization procedures may be found in Section A-3.

The sterilized inflorescences were cultured by delicately planting the peduncle in agar (Fig. 5) or by inserting the peduncle between the side of the bottle and the cotton ball (Fig. 6).

4. Cultural Conditions

The cultures were incubated in growth chambers at different temperatures and under various light regimes. Details are given in Table XXI. Illumination for the cultures was provided by fluorescent tubes (Vita Lite, Duro-Test Electric Ltd.) with a light intensity of 21.1 μE $sec^{-1}m^{-2}$ (ca. 1,000 lux).

TABLE XXI. Light regimes and temperatures used for the culture of *Lotus* inflorescences

Treatment	Duration of regime	Photoperiod		Temperature	
		day (h)	night (h)	day (°C)	night (°C)
a	Continuous	24	0	25	—
b	10 days, then	0	24	—	25
	continuous	24	0	25	—
c	10 days, then	0	24	—	30
	continuous	24	0	25	—
d	10 days, then	24	0	30	—
	continuous	24	0	25	—
e	10 days, then	0	24	—	4
	continuous	24	0	25	—

5. Examination of the Cultures

After one or two months in culture, the inflorescences were examined for the presence of callus tissue on the anthers or the ovaries. Non-callused anthers were squashed in aceto-carmine, as described earlier (Section A-7-a), to determine whether multinucleate or multicellular microspores had been induced. Ovaries were dissected for an examination of the ovules.

F. Polyembryony

1. Material

Ten L. corniculatus germplasm sources and two diploid Lotus species, L. pedunculatus Cav. var. villosus and L. tenuis Waldst. et Kit., were used in these studies (Table XXII).

2. Preparation of the Seeds

Seeds were scarified with a piece of sandpaper and placed in 9-cm plastic petri dishes between two sheets of moistened filter paper. The petri dishes were incubated in the dark, either at room temperature in a laboratory bench drawer or at 25°C in a seed germination cabinet. When necessary, the petri dishes were watered with distilled water in order to prevent dessication of the seedlings.

3. Examination of the Seedlings

After five to six days, seedlings with cotyledons still enclosed in the seed coat were examined and counted. When a seedling appeared to possess two radicles, the seed coat was removed with dissecting needles to expose the cotyledons. In the case of twins, the seedlings were separated and either prepared for cytological examination or

TABLE XXII. *Lotus* germplasm used in the screening of polyembryonic seeds

Genetics Laboratory accession number	Species	Source
Diploids (2n=12)		
B145	*L. tenuis* Waldst. et Kit.	U.S.D.A., Soil Conservation Service, Pleasanton (Calif.) USA. P-14496
B246	*L. pedunculatus* Cav. var. *villosus* Lamotte	Rhône, Etablissements Loras, France. Groupement national interprofessionnel de production et d'utilisation des semences, graines et plants, Paris, France.
Tetraploids (2n=24)		
B247	*L. corniculatus* L.	Loir-et-Cher, Union coopérative agricole de Loir-et-Cher, France. Groupement national interprofessionnel de production et d'utilisation des semences, graines et plants, Paris, France
B249	*L. corniculatus* L.	Gers (Montaut), Etablissements Saint-Jeannet et Fils, France. Groupement national interprofessionnel de production et d'utilisation des semences, graines et plants, Paris, France.
B250	*L. corniculatus* L.	Gers (Vic-Fezensac), Etablissements Saint-Jeannet et Fils, France. Groupement national de production et d'utilisation des semences, graines et plants, Paris, France.

Continued...

TABLE XXII. (Continued)

Genetics Laboratory accession number	Species	Source
B251	L. corniculatus L.	Poitou, Établissements Louis Ricoux, France. Groupement national interprofessionnel de production et d'utilisation des semences, graines et plants, Paris, France.
B257	L. corniculatus L. cv. Viking	Commercial certified seed. Plant Breeding Station, Geneva (N.Y.), USA.
B325	L. corniculatus L. cv. Empire	Commercial certified seed. Plant Breeding Station, Geneva (N.Y.), USA.
B764	L. corniculatus L. cv. Leo	Commercial certified seed. Plant Science Department, Macdonald College, Ste-Anne de Bellevue (Que.), Canada.
B779	L. corniculatus L. cv. Mirabel	Commercial certified seed. Plant Science Department, Macdonald College, Ste-Anne de Bellevue (Que.), Canada
B780	L. corniculatus L.	Northrup, King & Co., Minneapolis (Minn.), USA. KO-4.
B781	L. corniculatus L. cv. Kimey	Origin, Chile. U.S.D.A., Beltsville (Md.), USA.

planted in Pro-Mix and transferred to the greenhouse to allow development.

4. Cytological Examination of Twin Seedlings

Determination of the chromosome number was performed on mitotic tissues (root tips). The root tips were fixed in Carnoy's fluid for 12 to 16 hours, rinsed several times with distilled water, and stained according to a standard Feulgen procedure. The root tips were then rinsed with distilled water and macerated in a 4% solution of pectinase for two to three hours. The root tips were again rinsed with distilled water and transferred to 45% acetic acid. Slides were prepared following a routine squashing technique.

III

RESULTS

Since no differences in the callusing response were found between anthers cultured with or without the ovary (and vice-versa), the results obtained with combined anther + ovary cultures have been presented separately in Sections A and D, respectively.

A. Anther Culture

The results obtained with the different culture media utilized are presented in the following section.

1. Murashige and Skoog (1962), Type I

a) Effect of the Growth Regulators CM and 2,4-D

The formation of a callus tissue was not induced on L. cruentus anthers cultured on the basic MS medium or the same medium supplemented with either different concentrations of coconut milk or a combination of 2,4-D and coconut milk (Table XXIII). The addition of activated charcoal to the various medium subtypes did not have any apparent effect on callus formation.

On all medium subtypes, the anthers, initially greenish, slowly turned brown and became necrotic. Cytological examination showed that most microspores had died; multinucleate and multicellular structures were not observed.

TABLE XXIII. Callus formation on L. cruentus anthers (Stage 1) cultured on MS, Type I (continuous illumination, 25°C)

Medium subtype*	Activated charcoal**	Number of anthers cultured (A)	Number of callusing anthers (B)	Frequency of callus induction (B/A)
1	+	20	0	0.00
	-	20	0	0.00
2	+	20	0	0.00
	-	20	0	0.00
3	+	20	0	0.00
	-	20	0	0.00
4	+	20	0	0.00
	-	20	0	0.00

*See material and Methods, Table V
**With activated charcoal (+); without activated charcoal (-)

b) Effect of Cultural Conditions

The effect of different temperature and light treatments was studied using anthers of cultivar Leo cultured on basic MS (Type I). For each cultural condition, treatments were supplemented with activated charcoal for comparison (Table XXIV).

Callusing anthers were observed in one treatment, which consisted of incubating the cultures in light at 30°C, on a medium devoid of activated charcoal.

Examination of callusing and non-callusing anthers submitted to the various treatments showed mostly uninucleate microspores and dead grains; supplementary divisions were not observed.

TABLE XXIV. Effect of different cultural conditions and activated charcoal on callus formation on Leo (EMS764) anthers (Stage 2) cultured on MS, Type I (subtype 1*)

Treatment**	Activated charcoal***	Number of anthers cultured (A)	Number of callusing anthers (B)	Frequency of callus induction (B/A)
a	+	80	0	0.00 a,b****
	-	100	0	0.00 a,b
b	+	80	0	0.00 a,b
	-	60	3	0.05 a
c	+	60	0	0.00 a,b
	-	80	0	0.00 a,b
d	+	80	0	0.00 a,b
	-	140	0	0.00 b

*See Material and Methods, Table V
**See Material and Methods, Table XI
***With activated charcoal (+); without activated charcoal (-)
****Values followed by the same letter are not significantly different at the 0.05 level

2. Murashige and Skoog (1962), Type II

a) Effect of the Growth Regulators BN and 2,4-D and Cultural Conditions

Lotus angustissimus anthers cultured on two subtypes of MS medium (Type II) were submitted to different cultural conditions (Table XXV). The data in Table XXV show that, in general, the frequency of callus induction was low for all sets of conditions on both medium subtypes, which had been supplemented with 2,4-D and benazolin, or benazolin alone.

The addition of activated charcoal apparently inhibited callusing as callus formation was limited to anthers cultured on media

without charcoal. However, significant differences were not found either within the treatments or between them.

Non-callusing and callusing anthers were squashed to determine the degree of pollen development. Uninucleate microspores and dead grains were most prevalent; multinucleate or multicellular structures were not observed in any of the anthers examined.

TABLE XXV. Effect of different cultural conditions and activated charcoal on callus formation on L. angustissimus anthers (Stage 2) cultured on MS, Type II

Medium subtype*	Cultural conditions**	Activated charcoal***	Number of anthers cultured (A)	Number of callusing anthers (B)	Frequency of callus induction (B/A)
3	a	+	20	0	0.00 a****
		-	40	4	0.10 a
	c	+	30	0	0.00 a
		-	30	4	0.13 a
	e	-	10	2	0.20 a
4	a	-	20	0	0.00 a
	c	+	20	0	0.00 a
		-	20	0	0.00 a
	e	-	30	3	0.10 a

*See Material and Methods, Table VI
**See Material and Methods, Table XI
***With activated charcoal (+); without activated charcoal (-)
****Values followed by the same letter are not significantly different at the 0.05 level

b) Effect of Activated Charcoal and High Sucrose Concentration

The effect of activated charcoal and high sucrose concentration on callus induction was studied using L. alpinus (4x) anthers (Stage 3) cultured on MS Type II supplemented with 0.5 ppm 2,4-D and 8%, or 12% sucrose (Table XXVI).

As shown by the data in Table XXVI, callusing only occurred on the medium containing 3% sucrose without charcoal. The frequency of callus induction was rather low and no significant differences were found within and between treatments.

Microscopical examination of the callusing and the non-callusing anthers from the various treatments showed that most pollen grains were binucleate and that some had been germinating (Fig. 7). Multicelled pollen grains were not seen in any of the material examined.

TABLE XXVI. Effect of activated charcoal and sucrose concentration on callus formation on L. alpinus (4x) anthers (Stage 3) cultured on MS, Type II (continuous illumination, 25°C)

Sucrose concen.	Activated charcoal*	Number of anthers cultured (A)	Number of callusing anthers (B)	Frequency of callus induction (B/A)
8%	+	80	0	0.00 a**
	-	80	4	0.05 a
12%	+	80	0	0.00 a
	-	40	0	0.00 a

*With activated charcoal (+); without activated charcoal (-)
**Values followed by the same letter are not significantly different at the 0.05 level

3. Murashige and Skoog (1962), Type III

a) Effect of the Growth Regulators BAP and 2,4-D

Callus induction on liquid MS medium (Type III) was compared between two Lotus species (Table XXVII).

TABLE XXVII. Callus formation on anthers (Stage 2) cultured on liquid MS, Type III (continuous illumination, 25°C)

Species (Accession number)	Medium subtype*	Number of anthers cultured (A)	Number of callusing anthers (B)	Frequency of callus induction (B/A)
L. emeroides (B349)	1	140	40	0.28 a**
	2	80	36	0.45 b
	3	160	38	0.23 a,d
	4	120	54	0.45 b
L. mascaensis (B350)	1	100	5	0.05 e
	2	120	13	0.10 c,e
	3	180	28	0.15 c,d
	4	200	28	0.14 c,d

*See Material and Methods, Table VII
**Values followed by the same letter are not significantly different at the 0.05 level

The statistical analysis of the results obtained for L. emeroides anthers shows that the values for medium subtypes 1 and 3 or 2 and 4 were not significantly different, but that the values between these two groups (1-3 and 2-4) were significantly different. Since the culture media differed only by the growth regulator

supplementation, the results seem to indicate that 1) 1 ppm BAP was equivalent in effect to 1 ppm 2,4-D + 2 ppm BAP, 2) 2 ppm BAP was equivalent in effect to 2 ppm 2,4-D + 1 ppm BAP, and 3) the latter concentrations were significantly superior in promoting callus formation. In the case of L. mascaensis anthers, the level of callusing was low and only slight differences were noted.

Significant differences were found between the two Lotus species for medium subtypes 1 (1 ppm BAP), 2 (2 ppm BAP), and 4 (2 ppm 2,4-D + 1 ppm BAP).

The calluses originating from L. mascaensis anthers frequently assumed a spherical shape and were very compact with a more or less smooth surface (Fig. 8); these calluses produced chlorophyll when cultured under continuous illumination.

Calluses obtained from L. emeroides anthers grew more slowly and their shape was more irregular (Fig. 9).

Root formation took place on a few L. emeroides calluses subcultured on the differentiation medium (Fig. 10). Small and delicate plantlets were recovered (Figs. 11 and 12), but none survived transfer to soil; consequently, their ploidy could not be determined. Organogenesis was not observed on any of the calluses of L. mascaensis.

Cytological examination of non-callusing L. emeroides anthers from the four medium subtypes revealed the presence of multinucleate pollen grains. These multinucleate grains could not be found in any of the anthers of L. mascaensis.

Structures resembling multicellular pollen grains (Fig. 13) were observed on a few occasions among callus cells obtained from a callused L. emeroides anther cultured on medium subtype

4 (1 ppm BAP + 2 ppm 2,4-D).

b) Effect of a Cold Pretreatment

A cold pretreatment consisting of a 24-hour period at 4°C was applied to L. emeroides flower buds before culturing the anthers in order to attempt to stimulate the induction of pollen embryoids or callus formation. The results of this experiment are presented in Table XXVIII.

TABLE XXVIII. Effect of cold treatment on callus formation on L. emeroides anthers (Stage 2) cultured on liquid MS, Type III (continuous illumination, 25°C)

Medium subtype*	Cold treatment**	Number of anthers cultured (A)	Number of callusing anthers (B)	Frequency of callus induction (B/A)
1	+	120	22	0.18 a***
	-	140	40	0.28 a,d
2	+	100	59	0.59 b
	-	80	36	0.45 b
3	+	140	46	0.32 c
	-	160	38	0.23 c,d
4	+	160	75	0.46 b
	-	120	54	0.45 b

*See Material and Methods, Table VII
**With cold treatment (+); without cold treatment (-)
***Values followed by the same letter are not significantly different at the 0.05 level

The statistical analysis of the data revealed that there were significant differences between the different medium subtypes within treated anthers and within non-treated material. However, differences in the frequency of callusing were not found within any one medium

subtype between the treated and the untreated anthers. The cold treatment did not exert any particular influence on callus induction. However, the frequency of callus formation was enhanced by particular combinations of growth regulators, such as medium subtypes 2 and 4, containing 2 ppm BAP and 2 ppm 2,4-D and 1.0 ppm BAP, respectively, which gave the greatest response.

Pollen divisions leading to multinucleate structures were noted in both non-treated and treated anthers at about the same low frequency (0.01%). The calluses exhibited the same irregular appearance as described above. Structures resembling multicellular pollen grains (Fig. 14) were observed in calluses derived from cold-treated material cultured on medium subtype 3. Plantlets were recovered from calluses derived from both treated and untreated material which had been cultured on medium subtypes 3 and 4.

c) Effect of Agar

The effect of a solid vs. a liquid culture medium on callogenesis was studied on anthers of L. corniculatus cultured at the quartet stage (Stage 1).

The data presented in Table XXIX show that, for both medium subtypes, the frequency of callus induction was significantly greater in cultures inoculated on solid media. Significant differences were not found between solid medium subtypes, but the response of liquid medium 7 (1 ppm IAA + 2 ppm BAP) was significantly higher than on medium 6 (2 ppm IAA + 1 ppm BAP).

After two months of incubation, a heart-shaped embryo-like structure (Fig. 15) was observed to develop from an anther which had

TABLE XXIX. Effect of agar on callus formation on L. corniculatus (B259) (Stage 1) cultured on MS, Type III (continuous illumination, 25°C)

Medium subtype*	Agar**	Number of anthers cultured (A)	Number of callusing anthers (B)	Frequency of callus induction (B/A)
6	+	190	98	0.51 a***
	-	100	25	0.25 b
7	+	140	79	0.56 a
	-	130	50	0.38 c

*See Material and Methods, Table VII
**With agar (+); without agar (-)
***Values followed by the same letter are not significantly different at the 0.05 level

been cultured on medium subtype 6 (2 ppm IAA + 1 ppm BAP) solidified with agar. The green coloration of this structure contrasted sharply with the browning tissues of the anther and the remaining part of the filament which was also degenerating. The location of this structure suggested that it may have originated from the connective tissue. Similar structures could not be observed in the remainder of the anthers cultured under the same conditions.

The calluses were initially brownish and a few had spots of chlorophyllous tissue which were more compact than the surrounding tissues. Upon repeated subculturing, these islands of green tissue either became brown and soft or enlarged and, in some cases, produced shoots. Organogenesis was induced on calluses derived from anthers cultured on the two solid media. Root formation and the differentiation of plantlets (Fig. 16) were observed.

A few of these plantlets survived transfer to greenhouse conditions and were grown to maturity. One of the regenerants, which had been derived from anther culture on medium subtype 7 (1 ppm IAA + 2 ppm BAP), showed morphological differences with the donor plant. The mother plant (Fig. 17) possessed weak prostrate stems with long internodes and light green leaves, whereas the regenerated plant (Fig. 18) had robust erect stems with much shorter internodes and darker leaves. These differences were rather striking when both plants were placed side by side (Fig. 19). The plants presented some differences in the anthocyanin content of the stems and the pedicel and calyx of the florets. In the regenerant plant, a deep red coloration could be observed on the stems, whereas in the donor plant, the stems were entirely green. Anthocyanins were also present on the pedicel and the calyx of the florets of the regenerant plant, whereas in the donor plant, these parts were devoid of anthocyanins. Variation was also noted in the position of the keel. In the regenerated plant, the keel was completely enclosed by the wings (Fig. 20), whereas in the mother plant, the bottom part of the keel could be seen below the wings (Fig. 21). Differences in pubescence were also noticed. For example, few hairs were present on the calyx of the florets of the regenerated plant (Fig. 22), whereas on the calyx of the florets of the mother plant the pubescence was dense (Fig. 23). The margins of most leaflets of the regenerant were ciliate (Fig. 24), whereas, in the mother plant, the margins were glabrous (Fig. 25). Leaflet shape was generally similar in younger leaves; in more mature leaves taken from the mid portion fo the stem, the central leaflet had generally an acute tip

in the regenerant (Fig. 24), whereas that particular leaflet was rounder in the donor plant (Fig. 25).

An examination of the pollen mother cells revealed that there was a regular formation of bivalents at metaphase I; univalents and multivalents were rarely observed. There was also a relatively high percentage (ca. 10%) of anaphase I cells which showed one to four bivalents left at the equatorial plate (Figs. 26,27). This was reflected by the presence of one to several micronuclei (Fig. 28) in the quartets. However, pollen viability estimates indicated that both the donor plant and the regenerant had comparable values (89.4% and 83.6%, respectively). The regenerant, which was kept in the greenhouse under open-pollination conditions, produced seeds. About 30 pods (mean length 15.4 mm X mean width 2.6 mm) were harvested; these pods contained a mean number of 2.7 seeds/pod. There was approximately an equal number of plump and wrinkled seeds; the seed coat color ranged from uniformly brown to variously mottled.

Microscopical examination of the root tips indicated that the regenerant was a mixoploid. Metaphase cells showing the haploid number of chromosomes (Fig. 29) were recorded among a mixed population of predominantly tetraploid (Fig. 30) and octoploid cells (Fig. 31).

4. Murashige and Skoog (1962), Type II vs. Type III

The callusing frequencies of anthers of L. emeroides cultured on liquid Murashige and Skoog's medium, Type II or Type III, were compared. Both culture media were supplemented with 2 ppm BAP and 3% sucrose, and they differed only by the concentrations of the

vitamins and the myo-inositol, which were either used as formulated (Type II) or reduced by half (Type III).

TABLE XXX. Callus formation on anthers of L. emeroides (Stage 2) cultured on MS, Type II or Type III (continuous illumination, 25°C)

Medium*	Number of anthers cultured (A)	Number of callusing anthers (B)	Frequency of callus induction (B/A)
Type II	220	81	0.36 a**
Type III	80	36	0.45 a

*See Material and Methods, Section A-3-a
**Values followed by the same letter are not significantly different at the 0.05 level

As shown by the data in Table XXX, there were no significant differences between the values obtained for the two culture media. Multinucleate (up to 5 nuclei) pollen grains were observed in the material examined from both treatments.

5. Gamborg (1970)

Anther culture of three species of Lotus was attempted on Gamborg's culture medium supplemented or not with growth regulators (Table XXXI). The anthers were cultured at different stages of development.

The data presented in Table XXXI show that L. glaucus anthers produced callus on three medium subtypes out of the six tested. Significant differences were found between medium subtype 5

TABLE XXXI. Callus formation on anthers cultured on different subtypes of Gamborg's culture medium (16h–8h photoperiod, 25°C)

Species (Accession number)	Stage	Medium subtype*	Number of anthers cultured (A)	Number of callusing anthers (B)	Frequency of callus induction (B/A)
L. glaucus					
(B348)	3	1	20	10	0.50 a
		2	20	0	0.00 b
		3	10	0	0.00 b
		4	20	10	0.50 a
		5	10	10	1.00 c
		6	30	0	0.00 b
L. alpinus (4x)					
(T77)	2	7	10	0	0.00 b
		8	10	0	0.00 b
(L. japonicus X L. alpinus)^2					
(A/JA)	3	1	20	10	0.50 a
		2	20	0	0.00 b

*See Material and Methods, Table VIII
**Values followed by the same letter not significantly different at the 0.05 level

(1 ppm 2,4-D + 1.0 α,β-D + 0.1 ppm K) which gave the best response, medium subtypes 1 and 4 (basal and 1 ppm 2,4-D + 0.1 ppm, K respectively) which gave a similar lower response, and the other media which failed to induce callus formation. Anthers of L. alpinus did not produce calluses on any of the two medium subtypes used. Anthers of the amphidiploid (L. japonicus X L. alpinus)^2 produced callus on the basal medium

but none on the medium supplemented with 1 ppm α,β-D, a response similar to L. glaucus anthers cultured on these same media.

In all species, calluses were brownish and their texture was similarly irregular and soft regardless of the medium subtype utilized. Six plantlets were recovered from (L. japonicus X L. alpinus)^2 calluses subcultured on the differentiation medium. These plantlets did not survive transfer to soil. Organogenesis was not triggered in subcultured calluses of the two other Lotus species.

Cytological examination of non-callusing anthers provided no evidence of development of multinucleate and multicellular microspores.

6. Veliky and Martin (1970)

Anther culture of two Lotus species, L. emeroides and L. corniculatus cv. Mirabel, was attempted on Veliky and Martin's culture medium (Table XXXII). The anthers were inoculated at the uninucleate stage on the medium which was supplemented or not with growth regulators.

TABLE XXXII. Callus formation on anthers (Stage 2) cultured on Veliky and Martin's culture medium (continuous illumination, 25°C)

Species (Accession number)	Medium subtype*	Number of anthers cultured (A)	Number of callusing anthers (B)	Frequency of callus induction (B/A)
L. emeroides (B349)	1	30	19	0.63 a,b**
	3	70	40	0.57 a,b
	4	30	30	1.00 c

Continued...

TABLE XXXII. Continued.

Species (Accession number)	Medium subtype*	Number of anthers cultured (A)	Number of callusing anthers (B)	Frequency of callus induction (B/A)
L. corniculatus (AA779)	3	60	28	0.46 a
	4	120	77	0.64 b

*See Material and Methods, Table IX
**Values followed by the same letter are not significantly different at the 0.05 level

The data presented in Table XXXII indicate that the frequency of callus induction on VM medium is relatively high on all subtypes (> 40%). The statistical analysis of the data shows that L. emeroides anthers produced significantly more callus on medium subtype 4 (0.5 ppm NAA) than on medium subtypes 1 or 3 (basal VM or supplemented with 2 ppm IAA, 1 ppm NAA,and 0.2 ppm K, respectively). Results obtained with L. corniculatus cv. Mirabel show that callus formation on medium subtype 4 was significantly higher than on medium subtype 3. However, the values obtained for both species on medium subtype 4 were significantly different, L. emeroides anthers showing a greater callus induction frequency.

Microscopic examination of the non-callusing anthers of both species from all treatments revealed that the microspores had degenerated. Multinucleate and multicellular grains were not observed.

The calluses which were brownish, loosely textured and slow growing, were apparently proliferating from the surface of the anthers. Due to a low mitotic index (≅ 0.01%), few metaphase cells were observed and these showed a tetraploid number of chromosomes. Cells with a

reduced chromosome number could not be found.

7. Potato Culture Medium

a) Effect of the Potato Extract and the Growth Regulators 2,4-D and K

Anther culture on potato media was attempted in three Lotus species. The results of these experiments are presented in Table XXXIII.

TABLE XXXIII. Callus formation on anthers (Stage 2) cultured on potato media (continuous light, 25°C)

Species (Accession number)	Medium subtype*	Number of anthers cultured (A)	Number of callusing anthers (B)	Frequency of callus induction (B/A)
L. tenuis (B340)	1	70	0	0.00 a**
	2	40	12	0.30 b
L. japonicus (4x) (T129)	2	90	46	0.51 c
	4	90	12	0.13 a
L. corniculatus cv. Leo (AA764)	1	140	0	0.00 a
	2	100	35	0.35 b
	4	90	1	0.01 a

*See Material and Methods, Table X
**Values followed by the same letter are not significantly different at the 0.05 level

The data first show that the medium without added growth regulators was not conducive to callus formation in the two species assayed. In L. tenuis, the frequency of callus induction of medium subtype 2 (2 ppm 2,4-D + 0.5 ppm K) was significantly greater than on the unsupplemented medium. This was also found in L. corniculatus.

Significant differences were also noted between the values obtained on medium subtypes 2 (2 ppm 2,4-D + 0.5 ppm K) and 4 (2 ppm 2,4-D) for L. japonicus and, similarly, for L. corniculatus. In general, for all three species, the response on medium subtype 2 was significantly greater than on any other medium used. Also, on this medium a significantly greater number of anthers from L. japonicus than from the other two species formed calluses.

The calluses obtained from these experiments were generally green or brown with islands of green tissue. The calluses which were subcultured gradually lost their green coloration but usually regained it after being transferred to fresh medium. Organogenesis was induced only on L. japonicus calluses. Roots and plantlets were observed in a few of the cultures. A cytological investigation could not be performed because the cultures were lost following the accidental failure of the cooling system of the incubator.

Microscopical examination of the non-callusing anthers from all cultured species showed that either development of the microspores had not taken place or that the microspores had undergone the first pollen mitosis and had become binucleate.

Examination of anthers at an early stage of callusing revealed that the callus was originating from either the anther wall, the connective tissue, or the remaining part of the filament. Evidence of the presence of pollen calluses could not be found.

Anther culture of L. villosus was also attempted on potato medium. The anthers were inoculated at the first pollen mitosis on the medium supplemented with 2 ppm 2,4-D and 0.5 K. Out of 80 anthers cultured, 20 produced callus, which gives a frequency of callus induction of 25%.

Anthers at an early stage of callusing were squashed to examine pollen development. Binucleate microspores showing two symmetrical, equally-staining, nuclei (Fig. 32) were observed in the cultures, as well as binucleate asymmetrical grains (Fig. 33). These were morphologically similar to the binucleate pollen grains found in L. villosus (Fig. 34), that is, with a crescent-shaped, darkly staining, generative nucleus, and a round, more diffuse, vegetative nucleus. Trinucleate grains, composed of one large vegetative-like nucleus and two darkly staining generative-like nuclei were also observed (Fig. 35). Pollen grains with four nuclei (Fig. 36) were occasionally seen. Multicellular structures (Figs. 37, 38) were observed at an extremely low frequency (ca. 0.01%). Further stages of development were not found.

Multinucleate grains were also observed in the non-callusing anthers.

b) Effect of Activated Charcoal

The effect of activated charcoal added to potato media on callus formation was studied using anthers of three Lotus species. The results of these experiments are presented in Table XXXIV.

From the data in Table XXXIV, it can be seen that the callusing response was, in general, low on all media. Significant differences between regular and charcoal-plus media were found in two species, L. angustissimus and L. tenuis, cultured on medium subtype 3 (2 ppm 2,4-D + 1 ppm BN + 0.5 ppm K).

Examination of the non-callusing anthers provided no evidence for the development of pollen calluses or embryoids.

TABLE XXXIV. Effect of activated charcoal on callus formation on anthers (Stage 3) cultured on potato media (continuous illumination, 25°C)

Species (Accession number)	Medium subtype*	Activated charcoal**	Number of anthers cultured (A)	Number of callusing anthers (B)	Frequency of callus induction (B/A)
L. angustissimus (B141)	2	+	20	0	0.00 a***
		-	30	0	0.00a
	3	+	30	0	0.00 a
		-	20	5	0.25 b,c
L. tenuis (B340)	2	+	40	0	0.00 a
		-	40	4	0.10 a
	3	+	30	5	0.16 a,b
		-	20	11	0.55 c
L. emeroides (B349)	2	+	50	0	0.00 a
		-	60	0	0.00 a
	3	+	50	1	0.02 a
		-	60	0	0.00 a

*See Material and Methods, Table X
**With activated charcoal (+); without activated charcoal (-)
***Values followed by the same letter are not significantly different at the 0.05 level

B. Pollen Culture

Experiments designed to culture isolated microspores or pollen grains proved totally unsuccessful in the two species cultured. The microspores of L. corniculatus degenerated very rapidly and were unable to pursue their development. Mature pollen grains of L. emeroides were still living after three months of incubation at 25 or 30°C, as

demonstrated by the grains which were taking up the stain (Fig. 39). However, no further development of the pollen was induced in the cultures and germination of the grains was not observed.

C. Pollen Tube Culture

After one month of incubation, macroscopic callus tissues could not be observed in any of the species cultured (Table XXXV).

TABLE XXXV. Pollen tube culture on MS culture medium supplemented with 3% sucrose and 2 ppm 2,4-D

Species (Accession number)	Callus formation
L. corniculatus (B259)	-
L. cruentus (B79)	-
L. mascaensis (B350)	-
L. pedunculatus (B124)	-
(L. krylovii X L. tenuis)^2 (A/[illegible])	-

Microscopic examination of the cultures did not show any evidence for the induction of either multinucleate or multicellular pollen tubes. In some cases, pollen tube development appeared to have been effected by the treatment, as illustrated by the presence of branching tubes (Fig. 40) and some extremely large tubes (Fig. 41).

D. Ovary and Ovule Culture

1. Murashige and Skoog (1962), Type I

a) Effect of the Growth Regulators Z and IAA

Ovaries and ovules of several Lotus species cultured at different stages of development on basal MS medium or on MS supplemented with IAA or Z generally failed to produce calluses (Table XXXVI). Callusing ovaries and ovules were obtained, however, with one genotype of L. corniculatus. The explants were cultured on a medium supplemented with zeatin (0.1 ppm) and IAA (0.1 ppm). There was no significant difference in the frequency of callus induction of stage 2 and 3 ovaries cultured on medium subtype 4 (0.1 ppm Z and 0.1 ppm IAA).

TABLE XXXVI. Callus formation on ovaries cultured on MS, Type I (16h-8h photoperiod, 25°C)

Species (Accession number)	Stage	Medium subtype*	Number of ovaries cultured (A)	Number of callusing ovaries (B)	Frequency of callus induction (B/A)
L. krylovii (B86)	2	1	6	0	0.00
L. corniculatus (B280)	2	2	10	0	0.00
L. corniculatus (B285)	3	2	3	0	0.00
L. corniculatus (B534)	2	1	2	0	0.00
		3	12	0	0.00
		4	13	12**	0.92

Continued...

TABLE XXXVI, Continued.

Species (Accession number)	Stage	Medium subtype*	Number of ovaries cultured (A)	Number of callusing ovaries (B)	Frequency of callus induction (B/A)
	3	4	5	5**	1.00
L. corniculatus (B554)	2	1	10	0	0.00
	3	1	7	0	0.00
		3	4	0	0.00

*See Material and Methods, Table XV
**Callusing ovules

The brownish ovary calluses originated from the wall tissues. There was no evidence for the development of the ovules that were left inside. Ovule calluses did not develop sufficiently to permit a cytological investigation.

b) Effect of the Growth Regulators CM and 2,4-D

The effect of supplementing the basal MS medium with coconut milk and 2,4-D was studied using L. cruentus ovaries. The effect of activated charcoal in the different medium subtypes was also assayed. The various media proved totally inconducive in inducing callus from the ovaries (Table XXXVII). In all instances, the ovaries rapidly turned brown and became wrinkled and necrotic. Development of the young ovules did not proceed.

TABLE XXXVII. Callus formation on L. cruentus ovaries (Stage 1) cultured on MS, Type I (continuous illumination, 25°C)

Medium subtype*	Activated charcoal**	Number of ovaries cultured (A)	Number of callusing ovaries (B)	Frequency of callus induction (B/A)
1	+	2	0	0.0
	-	2	0	0.0
5	+	2	0	0.0
	-	2	0	0.0
6	+	2	0	0.0
	-	2	0	0.0
7	+	2	0	0.0
	-	2	0	0.0

*See Material and Methods, Table XV
**With activated charcoal (+); without activated charcoal (-)

c) Effect of Cultural Conditions

A study was conducted to determine the influence on callus formation from the ovaries of L. corniculatus cv. Leo at two incubation temperatures, 25°C and 30°C. The temperature treatments were either applied continuously or over a period of ten days, in light, or in darkness. The presence of activated charcoal in the culture medium (basal MS) was also assayed.

As may be seen by the data in Table XXXVIII, callusing of the ovaries did not take place in any of the treatments regardless of the presence of activated charcoal in the culture medium.

The initial swelling of the explants was followed by a gradual discoloration and necrosis. Dissection of a few of these ovaries

showed the presence of abortive ovules.

TABLE XXXVIII. Effect of different cultural conditions and activated charcoal on callus formation on Leo (EMS764) ovaries (Stage 2) cultured on MS, Type I (Subtype 1)

Cultural conditions*	Activated charcoal**	Number of ovaries cultured (A)	Number of callusing ovaries (B)	Frequency of callus induction (B/A)
b	+	8	0	0.0
	-	12	0	0.0
c	+	8	0	0.0
	-	12	0	0.0
d	+	8	0	0.0
	-	12	0	0.0
e	+	8	0	0.0
	-	12	0	0.0
f	+	8	0	0.0
	-	8	0	0.0

*See Material and Methods, Table XVIII
**With activated charcoal (+); without activated charcoal(-)

2. Murashige and Skoog (1962), Type II

The effect of two high sucrose concentrations was tested on L. alpinus (4x) mature ovaries. The explants were cultured on MS, Type II, supplemented with 0.5 ppm 2,4-D; the effect of activated charcoal was also studied.

The data in Table XXXIX show that calluses were not produced in media supplemented with activated charcoal. Callusing took place in the media without charcoal, but there was no significant difference between the two sucrose concentrations in the frequency of callus induction.

TABLE XXXIX. Effect of sucrose and activated charcoal on callus formation on L. alpinus (4x) mature ovaries cultured on MS, Type II with 0.5 ppm 2,4-D (continuous illumination, 25°C)

Sucrose concentration	Activated charcoal*	Number of ovaries cultured (A)	Number of callusing ovaries (B)	Frequency of callus induction (B/A)
8%	+	8	0	0.00 a**
	-	8	4	0.50 a
12%	+	8	0	0.00 a
	-	5	1	0.20 a

*With activated charcoal (+); without activated charcoal (-)
**Values followed by the same letter are not significantly different at the 0.05 level

The non-callusing ovaries gradually turned brown and became necrotic. The isolated ovules were not observed to callus on any of the media.

3. Murashige and Skoog, Type III

a) Effect of the Growth Regulators BAP and 2,4-D

The callusing response of ovaries of two Lotus species was compared on liquid MS medium Type III (Table XL).

As shown by the results presented in Table XL there was a generally high induction level in both species. The analysis of the data showed that there were no significant differences within species between the medium subtypes used, and between species for any one medium subtype, except in the case of medium 1 where a comparison was not made.

TABLE XL. Callus formation on Lotus ovaries (Stage 2) cultured on liquid MS, Type III (continuous illumination, 25°C)

Species (Accession number)	Medium subtype*	Number of ovaries cultured (A)	Number of callusing ovaries (B)	Frequency of callus induction (B/A)
L. emeroides (B349)	2	12	11	0.92 a**
	3	18	18	1.00 a
	4	12	11	0.92 a
	5	12	10	0.83 a
L. mascaensis (B350)	1	24	22	0.91 a
	2	8	8	1.00 a
	3	9	9	1.00 a
	4	12	10	0.83 a
	5	12	8	0.66 a

*See Material and Methods, Table XVI
**Values followed by the same letter are not significantly different at the 0.05 level

In both species, green, white, and brown calluses with a rather compact texture were observed to originate from different areas of the ovary (Figs. 44, 45). When subcultured, the calluses gradually lost their coloration, turning brownish and becoming watery. Organogenesis was limited to a few roots developing from one callus of L. emeroides which had been initiated on an ovary cultured on medium subtype 5 (2 ppm 2,4-D + 1 ppm BAP). Eight root tips, approximately 1 to 3 cm in length, were examined for chromosome number. The mitotic rate was low and only 21 prophase-metaphase figures were observed from an estimated 4,000 cells examined. Both euploid (4 cells, 2n=14; 12

cells, 2n=28; and 1 cell, 2n=56) and aneuploid (3 cells, 2n=14-28; and 1 cell, 2n=16) cells were observed. The haploid cells (2n=14) were found in a single root tip along with tetraploid cells (2n=28). Several root tips were mixoploid with subtetraploid numbers between 14 and 28. The octoploid cell (2n=56) was found in a root tip in which a tetraploid cell (2n=28) was also observed.

Shoots and plantlets were not produced from any of the subcultured calluses of both species. Dissection of the non-callusing ovaries did not provide any evidence of development of the ovules.

b) Effect of a Cold Pretreatment

The effect of a cold treatment on the induction of parthenogenesis and callus formation was studied on ovaries of L. emeroides. The explants were cultured on a modified liquid MS medium supplemented with various concentrations of BAP and/or 2,4-D.

TABLE XLI. Effect of a cold treatment on callus formation on L. emeroides ovaries (Stage 2) cultured on MS, Type III (continuous illumination, 25°C)

Medium subtype*	Cold treatment**	Number of ovaries cultured (A)	Number of callusing ovaries (B)	Frequency of callus induction (B/A)
2	+	10	9	0.90 a***
	-	12	11	0.92 a
3	+	12	11	0.92 a
	-	18	18	1.00 a
4	+	12	11	0.92 a
	-	12	11	0.92 a

Continued...

TABLE XLI. Continued.

Medium subtype*	Cold treatment**	Number of ovaries cultured (A)	Number of callusing ovaries (B)	Frequency of callus induction (B/A)
5	+	18	16	0.89 a
	-	12	10	0.83 a

*See Material and Methods, Table XVI
**With cold treatment (+); without cold treatment (-)
***Values followed by the same letter are not significantly different at the 0.05 level

The data in Table XLI indicate that a high frequency of callus induction was reached on every medium subtype, with or without a cold treatment. There were no significant differences between any of the media used and within any one medium between treated and non-treated material.

On all medium subtypes the calluses which developed were green and relatively firm and proliferated on the surface of the explant (Fig. 45). Dissection of non-callusing ovaries and non-responsive portions of callusing ovaries showed only abortive ovules, which did not elicit callus formation.

The calluses gradually turned brown upon repeated subculturing and became more loosely textured and watery. Organogenesis was limited to a few roots as described previously. Shoot and plantlet formation did not procced on any of the subcultured material.

c) Effect of Agar

The effects of solid vs. liquid culture media on the frequency of callus induction were compared using young ovaries of L. corniculatus (B259).

TABLE XLII. Effect of agar on callus formation on L. corniculatus (B259) ovaries (Stage 1) cultured on MS, Type III (continuous illumination, 25°C)

Medium subtype*	Agar**	Number of ovaries cultured (A)	Number of callusing ovaries (B)	Frequency of callus induction (B/A)
6	+	8	8	1.00 a***
	-	3	2	0.66 a
7	+	18	18	1.00 a
	-	4	3	0.75 a

*See Material and Methods, Table XVI
**With agar (+); without agar (-)
***Values followed by the same letter are not significantly different at the 0.05 level

As shown by the data in Table XLII, the frequency of callus induction was high in all cases, but significant differences were not found between solid and liquid medium for both medium subtypes utilized.

Fast-growing green calluses (Fig. 42) were obtained from all treatments, but gradually turned brown and grew more slowly on the differentiation medium. Shoot formation (Fig. 43) was observed from subcultured calluses derived from ovaries cultured on solid medium subtype 7 (1 ppm IAA and 2 ppm BAP).

Ovules were not explanted from the ovaries due to their small size at this stage of development. Only uncallused degenerating ovules were observed in the cultured ovaries.

4. Gamborg (1970)

Ovaries from several species of Lotus were dissected at various

stages of development and cultured on basal Gamborg's medium or on this medium supplemented with different growth regulators.

As can be seen by the data in Table XLIII, calluses were induced on most ovaries from all the species cultured, regardless of the culture medium used and the stage of development of the explants.

TABLE XLIII. Callus formation on ovaries and ovules cultured on different subtypes of Gamborg's medium (16h-8h photoperiod, 25°C)

Species (Accession number)	Stage	Medium subtype*	Number of ovaries cultured (A)	Number of callusing ovaries (B)	Frequency of callus induction (B/A)
L. glaucus (B348)	2	1	1	1	1.00
		3	2	2	1.00
		4	1	1	1.00
		6	3	3	1.00
		7	1	0	0.00
		8	3	3	1.00
	3	1	3	2	0.66
		3	5	4	0.80
		4	2	2	1.00
		5	2	1	0.50
		7	3	3	1.00
		8	3	3	1.00
L. alpinus (4x) (T77)	1	1	1	1	1.00
		9	1	1	1.00
		10	1	1	1.00
		11	1	1	1.00
	2	9	5	4**	1.00

Continued...

TABLE XLIII. Continued.

Species (Accession number)	Stage	Medium subtype*	Number of ovaries cultured (A)	Number of callusing ovaries (B)	Frequency of callus induction (B/A)
		10	5	3	0.60
		11	4	4	1.00
	3	1	1	1	1.00
		8	3	3	1.00
		9	3	3	1.00
		10	2	2**	1.00
		11	1	1	1.00
(*L. japonicus* X *L. alpinus*)^2 (A/JA)	2	1	2	2	1.00
		3	2	1	0.50
	3	1	1	1	1.00
		4	1	1	1.00
		6	1	1	1.00
		7	1	1	1.00
		8	1	1	1.00
(*L. japonicus* X *L. alpinus*)^2 X *L. corniculatus* (JA-2-15)^2 X 554	3	2	8	5	0.63
(*L. krylovii* X *L. schoelleri*)^2 X *L. corniculatus* (KS 7(a)5)^2 X 554	2	6	1	1	1.00
	3	4	1	1	1.00

*See Material and Methods, Table XVII

**Callusing ovules

Callusing ovules were observed in cultures of L. alpinus on medium subtypes 9 and 10, supplemented with 0.5 ppm 2,4-D and 1.0 ppm BAP, and 0.5 ppm NAA and 1.0 ppm BAP, respectively. The ovular calluses seemed to arise from the outer integument and did not grow sufficiently to allow for a cytological investigation.

The ovary calluses developed from the surface of the ovary, being usually initiated at the cut end of the explant. Only abortive ovules were seen upon dissection of the callusing and the non-callusing ovaries.

Root formation occurred in one callus of the Lotus hybrid KS 7(a)5; this culture became contaminated, preventing the examination of the root tips.

Plantlets were obtained from calluses of the amphidiploid A/JA (Fig. 46) derived from Stage 2 and 3 ovaries placed on medium subtypes 1 (basal) and 4 (1.0 ppm NAA and 0.1 ppm K), respectively. These plantlets died shortly after their transfer to greenhouse conditions.

5. Veliky and Martin (1970)

Ovaries (stage 2) of Lotus corniculatus cv. Mirabel were cultured on Veliky and Martin's culture medium supplemented with either IAA, NAA, and K (subtype 3), or NAA alone (subtype 4).

The data in Table XLIV show that the frequency of callus induction was relatively high on both medium subtypes, but no significant differences were found between the media.

The calluses which grew on the ovaries were brown and did not develop chlorophyll, even when subcultured on the differentiation medium. Organogenesis was not induced on any of the subcultured

calluses. The non-callusing ovaries shrivelled and turned brown. Internal development was not observed.

TABLE XLIV. Callus formation on L. corniculatus cv. Mirabel ovaries (Stage 2) cultured on Veliky and Martin's culture medium (continuous illumination, 25°C)

Medium subtype*	Number of ovaries cultured (A)	Number of callusing ovaries (B)	Frequency of callus induction (B/A)
3	12	8	0.66 a**
4	16	12	0.75 a

*See Material and Methods, Table IX
**Values followed by the same letter are not significantly different at the 0.05 level

6. Potato Culture Medium

a) Effect of the Potato Extract and the Growth Regulators 2,4-D and K

After two months of incubation, the induction of callus tissue was observed in all species cultured on medium subtypes 1, 2, and 4 (Table XLV), with the exception of L. villosus ovaries and ovules inoculated on the unsupplemented medium.

The frequency of callus induction on ovaries of L. villosus cultured on media 1 and 2 (basal and 2 ppm 2,4-D + 0.5 ppm K, respectively) was significantly lower than the values obtained for any other species cultured on these two media. No significant differences were established between the frequency of callusing of ovaries of L. corniculatus cv. Leo, L. tenuis, and L. japonicus cultured on the same media.

TABLE XLV. Callus formation on ovaries (Stage 2) cultured on potato media (continuous illumination, 25°C)

Species (Accession number)	Medium subtype*	Number of ovaries cultured (A)	Number of callusing ovaries (B)	Frequency of callus induction (B/A)
L. villosus (B189)	1	9	0	0.00 a**
	2	24	7	0.29 a
L. tenuis (B340)	1	8	6	0.75 b
	2	4	4	1.00 b
L. corniculatus cv. Leo (AA764)	1	8	4	0.50 b
	2	12	9	0.75 b
	4	12	8	0.66 b
L. japonicus (T129)	1	16	10	0.62 b
	4	8	7	0.87 b

*See Material and Methods, Table X
**Values followed by the same letter are not significantly different at the 0.05 level

b) Effect of Activated Charcoal

From the data shown in Table XLVI, it could be inferred that the addition of activated charcoal in the culture medium had a decreasing effect on the frequency of callus induction, but the statistical analysis of the data, although performed on rather low numbers of observations, revealed that there were no differences in the values obtained for the regular and the charcoal-plus media, for any of the species tested.

TABLE XLVI. Effect of activated charcoal on callus formation on ovaries (Stage 2) cultured on potato media (continuous illumination, 25°C)

Species (Accession number)	Medium subtype*	Activated charcoal**	Number of ovaries cultured (A)	Number of callusing ovaries (B)	Frequency of callus induction (B/A)
L. angustissimus (B141)	2	+	2	0	0.00 a***
		-	3	1	0.33 a
	3	+	3	0	0.00 a
		-	2	0	0.00 a
L. tenuis (B340)	2	+	4	1	0.25 a
		-	5	4	0.80 a
	3	+	4	1	0.25 a
		-	4	3	0.75 a
L. emeroides (B349)	2	+	5	1	0.20 a
		-	6	2	0.33 a
	3	+	5	0	0.00 a
		-	6	3	0.50 a

*See Material and Methods, Table X
**With activated charcoal (+); without activated charcoal (-)
***Values followed by the same letter are not significantly different at the 0.05 level

In general, the development of the callus on the potato media was limited to the base of the ovary (i.e. the cut surface), the remaining part of the ovary becoming brown and necrotic; in some cases, tissue was initiated from the entire ovary surface; callus, however, was never observed to arise from the stylar and the stigmatic tissues. The callus originated from the ovarian tissues and was not observed to develop on ovules either isolated on the culture medium or left inside the ovary. The isolated ovules usually turned brown and degenerated within the first few weeks of incubation.

Portions of calluses recovered from the different treatments were subcultured; initially green and rather compact, the calluses gradually lost their coloration, turned brown and became soft and watery. Plantlets could not be regenerated from any of the calluses subcultured on the differentiation medium.

E. In Vitro Culture of Inflorescences

In general, the cultured inflorescences did not show any morphological development; however, in mature florets (cultured when still unexpanded), the ovaries first increased in size and elongated through the floral parts, then finally turned brown and started degenerating (Fig 47).. Dissection of these ovaries and those of younger buds revealed that the ovules had shrunken and become necrotic.

Cytological examinations of anthers dissected from buds cultured on the different media and under the various cultural conditions showed that the microspores had remained at the particular developmental stage which preexisted at the initiation of the culture. Multinucleate and multicellular pollen grains were not found in the anthers of any of the species cultured.

While callus formation was not observed on the anthers or ovaries in the cultured inflorescences, callus tissue frequently developed from the cut end of the peduncle (Table XLVII).

Callusing of the peduncle occurred in solid and liquid culture media regardless of the cultural conditions used. In all species tested, the addition of activated charcoal to the culture medium completely suppressed the formation of callus tissue.

The development of roots from the peduncle was observed in cultures of L. angustissimus and the two Lotus hybrids when these cultures had

been submitted to a ten day dark period at 30°C (treatment c). Rooting was observed to occur only in solid medium of subtypes 2 and/or 3, regardless whether or not the medium had been supplemented with activated charcoal.

TABLE XLVII. Callus and organ formation from cultured Lotus inflorescences

Species (Accession number)	Medium subtype*	Cultural conditions**	Agar	Activated charcoal	Callus induction	Organ formation
L. angustissimus (B141)	1	a	+***	+	–	–
			+	–	+	–
		c	+	+	–	–
			+	–	+	–
		e	+	+	–	–
	2	a	+	+	–	–
			+	–	–	–
		c	+	–	–	R
			–	–	+	–
		d	+	+	–	–
			–	–	+	–
		e	–	–	–	–
	3	a	+	+	–	–
			+	–	+	–
		b	+	–	+	–
		c	+	+	–	R
			+	–	+	–
		d	–	–	+	–
		e	+	+	–	–
			+	–	–	–
L. alpinus (T77)	2	a	+	–	–	–
		c	+	–	–	–
		e	+	+	–	–
			+	–	–	–

Continued...

TABLE XLVII. Continued.

Species (Accession number)	Medium subtype*	Cultural conditions**	Agar	Activated charcoal	Callus induction	Organ formation
(L. burtii X L. filicaulis)[2] X L. corniculatus (303-F-2-3 X 554)	1	a	+	+	-	-
			+	-	-	-
		c	+	-	-	-
		e	+	+	-	-
	3	a	+	+	-	-
			+	-	+	-
			-	-	-	-
		c	+	+	-	R
			+	-	-	-
			-	-	-	-
		d	+	-	-	-
		e	+	+	-	-
			+	-	-	-
			-	-	+	-
(L. japonicus X L. alpinus)[2] X L. corniculatus (JA-10 X 554)	2	a	+	-	-	-
		b	+	-	-	-
		c	+	+	-	-
			+	-	-	R
		d	+	-	-	-
		e	+	+	-	-
			+	-	-	-

*See Material and Methods, Table XX
**See Material and Methods, Table XXI
***Presence (+); absence (-); Root (R)

F. Polyembryony

In the course of this study, a search for twin seedlings was made. The results are presented in Table XLVIII.

TABLE XLVIII. The occurrence of twin seedlings in Lotus species

Species			Seedlings examined	Twin seedlings
L. corniculatus				
	cv.	Empire	878	—
		Kimey	669	—
		Leo	12 447	—
		Mirabel	13 978	4
		Viking	2 835	—
	acc.	B247	1 439	—
		B249	902	—
		B250	1 507	—
		B251	873	—
		B780	4 305	—
L. tenuis			693	—
L. pedunculatus var. villosus			2 180	1

Twin seedlings were observed in two Lotus species, L. corniculatus cv. Mirabel and L. pedunculatus var. villosus. All twin pairs were composed of unequally sized members (Fig. 48).

The L. pedunculatus twin set degenerated within a few days after its transfer to soil, so that cytological data could not be obtained.

The first set of Mirabel twins was also lost soon after they were

planted in soil; the cotyledons were initially green and appeared normal, but there was no subsequent elongation of the epicotyl, and within three days the seedlings had shrivelled; consequently cytological examination of the seedlings could not be performed.

Transfer of the other Mirabel twins to soil was not attempted; the seedlings were fixed and stained soon after their recovery. Chromosome counts performed on the root tips of the seedlings revealed that they all possessed 24 chromosomes (Fig. 49), a number corresponding to the tetraploid condition in L. corniculatus. An octoploid cell (8x=48) was observed on one occasion among a population of tetraploid cells (Fig. 50). Haploid cells were not found in any of the twin pairs investigated.

IV

DICUSSION

A. Anther Culture

The culture of anthers was performed on three synthetic culture media, namely, the medium of Murashige and Skoog's (1962), originally designed for tobacco cell cultures, and the media of Gamborg (1970), and Veliky and Martin (1970), both of which were devised for cell cultures of legumes. In addition, a chemically undefined medium based on a potato extract was also utilized.

1. Murashige and Skoog (1962), Type I

a) Effect of the Growth Regulators CM and 2,4-D

The formation of callus from anthers of L. cruentus was not observed on Murashige and Skoog's medium whether, or not, it was supplemented with coconut milk alone, or in combination with 2,4-D. Coconut milk has been shown to contain several active compounds such as myo-inositol, 1,3-diphenylurea, a leucoanthocyanin, an arabinose ester of indoleacetic acid, IAA arabinose, zeatin, and zeatin ribose (Salisbury and Ross, 1969; Thimann, 1977), as well as minerals (Anonymous, 1976). Haploids were recovered from anther cultures on media supplemented with coconut milk, for example, Datura metel (Chandy and Narayanaswamy, 1971) and Solanum melongena (Raina and Iyer, 1973). Niizeki (1971) did not observe callus formation or pollen embryogenesis from anthers of L. corniculatus (both cvs.

Viking and Empire), L. corniculatus var. crassifolius, and L. japonicus cultured on Miller's medium (1963) supplemented with either 15% CM alone or 0.1 ppm IAA, 40 ppm AS, and 15% CM.

Anther culture of L. cruentus was also attempted by MacDonald (1974) on Gamborg's medium (1970) supplemented with 1.0 ppm K and 1.0 ppm NAA. Somatic callus was produced only on stage 3 anthers (that is containing binucleate pollen grains) at a frequency of 17.9%. He did not observe any embryogenesis in the pollen grains.

b) Effect of Cultural Conditions

Chinese researchers have observed that an incubation temperature of 30°C was superior to lower temperatures (23 to 26°C) for inducing callus from anthers of certain rice genotypes (Anonymous, 1974). Keller and Armstrong (1978) also recently reported that culture temperatures greater then 25°C drastically increased the number of embryos from Brassica napus anthers. According to the authors, even short treatments of one day at 30, 35, or 40°C stimulated embryo production. The highest embryo yields were obtained by culturing the anthers for two to three weeks at 30°C prior to transferring the embryos to 25°C. The authors suggested that high temperature might induce a switch in the microspores from their normal course of development. The treatment may cause a neutralization in their tendency to develop towards maleness as has been discussed by Pandey (1973). High temperature treatments at 30°C did not trigger the induction of pollen callus or pollen embryogenesis in cultured anthers of L. corniculatus cv. Leo (EMS764). Somatic callusing was not promoted by any of the treatments which seems to indicate that the medium and/or the cultural conditions were inappropriate. The

incubator temperature of 30°C was found inhibitory to both callus induction and growth in cultured anthers of Phaseolus vulgaris (Peters et al., 1977).

2. Murashige and Skoog (1962), Type II

a) Effect of the Growth Regulators BN and 2,4-D and Cultural Conditions

A higher temperature treatment (30°C), applied continuously or prior to transfer of the microspores to 25°C, was found ineffective in triggering pollen callusing or embryogenesis. These results must be considered inconclusive because of the small number of anthers cultured. Similarly, the apparent inhibition of callusing on medium subtypes supplemented with activated charcoal may be the result of the small number of anthers cultured.

The growth-promoting effect of auxins, including 2,4-D, has recently been reviewed (Thimann, 1977). While considerable information is known about these substances, their precise molecular mode of action remains unclear. Benazolin (4-chloro-2-oxobenzothiazolin-3-yl acetic acid) has been reported to possess auxin-like properties in that it can substitute for 2,4-D and NAA in culture media (Ingram and Butcher, 1972) and induce callus in species which do not respond to 2,4-D supplementation. It was concluded from the studies of Ingram and Butcher (1972) that benazolin could be of value in initiating callus from species and tissues, which have been found to be difficult to induce growth in otherwise. In L. angustissimus, the level of callusing was rather low regardless of the growth regulators used in the formation.

b) Effect of Activated Charcoal and Sucrose Concentration

The effects resulting from the addition of activated charcoal to culture media will be discussed below in Section A-7-b.

Higher levels of sucrose than commonly used (normally 2 to 3%, Sunderland, 1974) have been shown to promote the formation of pollen callus in cultured anthers of several cereals, such as barley (Clapham, 1973), wheat (Ouyang et al., 1973; Picard and de Buyser, 1973), and rice (Miao et al., 1978). In Brassica campestris sucrose concentrations below 6% did not result in embryoid production and increased levels inhibited somatic cell enlargement and callusing (Keller et al., 1975). In tobacco, higher sucrose levels exerted an adverse effect on embryogenesis resulting in the formation of albino plantlets (10.6% sucrose), callusing and production of aberrant albino plantlets (13.6% sucrose), and cessation of growth (17.1% sucrose) (Sharp et al., 1971). The highest level of callusing from tomato anthers was observed at 13.6% sucrose, but morphogenesis from these calluses only occurred following their transfer to a medium with a lower concentration of sugar (4%) (Sharp et al., 1971). Sucrose appears to play an important role, not solely as an osmotic agent, but also as an important metabolite (Miao et al., 1978).

Very little callusing took place on anther of L. alpinus cultured on a medium with high concentrations of sucrose and pollen embryogenesis was not triggered. It is possible that the stage of the pollen (stage 3) may not have been conducive to induction. Callus formation from anthers of L. corniculatus cv. Viking (stage 1, 2, and 3) cultured on Gamborg's medium supplemented with NAA and K and different sucrose concentrations (0, 3, and 12%) was not induced

(MacDonald, 1974).

3. Murashige and Skoog (1962), Type III

a) Effects of the Growth Regulators BAP and 2,4-D

The differences observed in the frequency of callus induction and organogenesis for the two species cultured may be attributed to genotypic differences, L. emeroides showing a greater response than L. mascaensis on three medium subtypes. Genotypic differences have been observed in several species including Lotus species cultured on the same media and submitted to identical cultural conditions (Niizeki, 1971; MacDonad, 1974).

The differences and the similarities in effect observed for the growth regulator formulations cannot be satisfactorily explained at this time.

b) Effect of a Cold Pretreatment

Cold pretreatments applied to flower buds and inflorescences prior to culturing the anthers have been reported to promote pollen embryogenesis or callusing in several species, such as Datura innoxia (Nitsch and Norreel, 1973; Sangwan-Norreel, 1977), tomato (Debergh and Nitsch, 1973), rice (Fouletier, 1974), wheat (Picard and de Buyser, 1975), rye (Wenzel et al., 1976b), and tobacco (Sunderland and Roberts, 1977a, 1979).

Temperature shocks applied during the first pollen mitosis have been reported to cause a reorientation of the spindle axis, an event which was sometimes followed by supernumerary divisions in the pollen grains (Sax, 1935). Nitsch and Norreel (1973) observed the same phenomenon in cultured anthers of Datura innoxia given a cold

pretreatment prior to culture. The spindle axis was modified which resulted in the production of two morphologically similar nuclei instead of the typical vegetative and generative nuclei. The authors proposed that the embryoids were derived from these abnormal pollen grains. This hypothesis has been challenged by the observation that embryoids can develop from pollen grains in which a typical asymmetrical division has taken place, as in henbane (Raghavan, 1976, 1978). Wenzel et al. (1976b, 1977a) concluded from their studies on rye that the cold treatment blocked the development of the microspores just prior to the first pollen mitosis, preventing the synthesis of starch. As a result, they considered there were a greater number of inducible microspores present at the time the microspores were initially cultured. Duncan and Heberle (1976) also proposed a similar explanation for the effect of a cold treatment on cultured tobacco anthers. The inducible stage during which pollen is switched to embryogenesis appears to be the post mitotic period up to the time in which starch is accumulated (Sunderland, 1974; Dunwell, 1978). The views of Wenzel et al. (1976b) and Duncan and Heberle (1976) are in agreement with those reported earlier by Picard and de Buyser (1975) who observed an "arresting" effect of the cold treatment on the microspores, regardless of their stage of development, whether uninucleate or binucleate, or whether the nuclei were morphologically similar or different in size in the binucleate grains. Recently, Sunderland and Roberts (1979) proposed that the cold pretreatment "exerts its effect not by altering the course of the first pollen division - - - but by delaying anther deterioration and thus assuring the survival of a greater proportion of the pollen grains

switched into embryogenesis".

In Lotus, cold pretreatment of the flower buds (48 hours at 4°C) has been reported to be ineffective in triggering pollen embryogenesis (MacDonald, 1974). In the present studies with L. emeroides the cold pretreatment did not appear to promote nuclear divisions in the microspores, since multinucleate pollen grains were found at a similar frequency in both treated and non-treated material. The occasional presence of structures resembling multicellular pollen grains observed in calluses derived from cold-treated anthers does not provide sufficient evidence to assign a positive effect to the treatment but warrants further investigation.

c) Effect of Agar

Increased frequencies of tobacco embryogenesis and barley callus formation have been reported when liquid culture media were substituted for solid media (Wernicke and Kohlenbach, 1976; Wilson et al., 1978b). Higher embryo yields were also obtained in Scopolia carniolica following the use of a liquid culture medium (Wernicke and Kohlenbach, 1975). It has been suggested that agar contains inhibitory substances (Wernicke and Kohlenbach, 1976) and the utilization of highly purified agar has been shown to minimize its adverse effects (Kohlenbach and Wernicke, 1978).

The level of callusing of L. corniculatus anthers was higher on solid media than on liquid media and, possibly, haploid callus tissue was derived from one anther cultured on solid medium, as discussed below (Section A-8) in relation to the mixoploid regenerant. The results obtained seem to indicate that somatic callusing and perhaps pollen callusing were favored by the addition of agar, which appears

to contradict the current literature. However, the possibility exists that inhibitory substances were still present in the material used to support the explants in spite of all the precautions taken.

The finding that significant differences in the frequency of callus formation existed between liquid medium subtypes but not between the equivalent solid media raises the question, if these differences are real, whether there exists a differential effect of the growth regulators when they are supplied to the explants in a liquid form rather than in agar media.

4. Murashige and Skoog (1962), Type II vs. Type III

Niizeki (1971) cultured anthers of several Lotus species on media with and without supplementation of vitamins and myo-inositol and obtained a wide range of callus formation. His results suggested that the vitamins and myo-inositol fraction exerted no influence on callus induction, but played an important role in callus growth.

The present studies with L. emeroides showed that decreasing the concentration of these constituents had no effect on callus induction, which could mean that this fraction was not needed for the induction of callus, as observed by Niizeki (1971). However, the possibility that the smaller amounts of vitamins and myo-inositol present in Type III had been active cannot be ruled out entirely.

Although multinucleate pollen grains were observed, multicellular structures could not be detected in the cultures, which suggests that the culture media may have been appropriate for inducing nuclear divisions, but not for allowing further development, even if the media were supplemented with a cytokinin (BAP).

5. Gamborg (1970)

MacDonald (1974) determined that Gamborg's culture medium (1970) was more conducive to callus formation from root cultures of L. corniculatus (cv. Viking) than media containing higher concentrations of nitrates and calcium, such as Murashige and Skoog's (1962) and Liau and Boll's (1970) culture media. Similarly, the results that he obtained from anther cultures of several Lotus species on either Gamborg's or Murashige and Skoog's culture media supplemented with the same growth regulators, indicated that a greater total frequency of callusing was attained on the former medium. In the present studies, callusing on Gamborg's medium was rather poor although the results must be considered with circumspection because of the small number of anthers explanted. No signs of pollen divisions were observed in any of the species cultured on Gamborg's medium, which agrees with the observation made earlier by MacDonald (1974).

6. Veliky and Martin (1970)

Peters et al. (1977) reported the presence of haploid cells in calluses derived from anthers of Phaseolus vulgaris inoculated on Veliky and Martin's culture medium (Appendix 3). Given the difficulties of chromosome number determination in callus tissues, the authors' illustrations of haploid chromosome complements are not overwhelmingly convincing. In the Lotus species studied on this medium, haploid cells were not observed in any of the calluses, nor was embryogenesis triggered in the microspores.

The level of callusing (somatic) was relatively high in spite of the small number of anthers cultured. This may relate to the observation by MacDonald (1974) that somatic callusing in species of

of Lotus is favored by the utilization of media containing relatively low concentrations of nitrates and calcium, such as that in Gamborg's medium (1970). Veliky and Martin's culture medium contains low concentrations of nitrates and calcium; this medium is a modification of Gamborg's medium PRL-4-C (1966), which is very similar to the 1970 formulation, but with a lower nitrate content. However, the VM medium is supplemented with a source of reduced nitrogen, casein hydrolysate, which may also promote callusing. In Lotus, the addition of casein hydrolysate has not proven beneficial to callus formation from cultured anthers of several species (Niizeki, 1971).

7. Potato Culture Medium

a) Effect of the Potato Extract and the Growth Regulators 2,4-D and K

A potato medium was successfully utilized by Chinese researchers (Anonymous, 1976) to improve the frequencies of pollen calluses in clutured anthers of wheat. Their analysis for a 20% extract per liter of the potato medium revealed the following: 1) there was approximately 1.4% sugar (glucose, fructose, and sucrose), 2) the level of nitrates was intermediate to those of Murashige and Skoog's (1962) and Miller's (1963) media, 3) the levels of phosphates and potassium were comparable to the N_6 medium (i.e. higher than Murashige and Skoog's medium), and 4) the level of ammonium was much lower than Murashige and Skoog's and Miller's culture media. The concentration of iron was estimated as being low.

Increased percentages of rye anthers undergoing androgenesis were obtained by Wenzel et al. (1977a) with the use of a potato

medium. Little information on the nature of the inductive substances is known. However, it is worth recalling that it has been recognized for some time that potato tubers contain inhibitory substances (anti-auxins?) which are able to antagonize the effects of coconut milk (Shantz et al., 1955). Furthermore, the observation that the addition of activated charcoal to the medium reduces its efficiency (Wenzel et al., 1977a) may mean that the active compounds are adsorbed and thus neutralized, as indicated by the findings of Constantin et al. (1977). Efforts are now being directed to the identification of the stimulatory compounds (Wenzel et al., 1977a).

In Lotus, the potato medium appeared to be effective only in L. villosus where multinucleate and multicellular structures were observed in the cultured anthers.

Two types of binucleate microspores were noticed in the cultures. The first type showed two symmetrical, equally-staining, generative-like nucleus, and a round, more diffuse, vegetative-like nucleus were observed. The binucleate grains belonging to the second type were morphologically similar to the mature pollen grains of L. villosus. Trinucleate grains observed in cultured L. villosus anthers were composed of one large vegetative-like nucleus and two darkly-staining smaller nuclei. Trinucleate grains similar to the ones described above were occasionally seen in the mature pollen of L. villosus, the smaller nuclei presumably being the sperm nuclei. This would constitute a case of premature second pollen mitosis, since, in Lotus, the generative nucleus divides in the pollen tube. Pollen grains with four nuclei(one large and

diffuse, and three darkly-staining and smaller) were also observed in the cultures, as well as structures which appeared multicellular.

The possible natural tendency of the generative nucleus of this species to divide prematurely may have been stimulated by the culture medium and lead to the formation of multicellular structures. It is interesting to note that pollen grains with more than two vegetative-like nuclei were not observed in any of the cultures. In most species investigated, the division of the generative nucleus does not play a significant role in the formation of embryoids, although, in the case of henbane, it has been claimed to be a major pathway to embryogenesis (Raghavan, 1976, 1978). It should be interesting to determine whether the responsive microspores belonged to a different population of pollen grains (pollen dimorphism) such as postulated by certain investigators (Hosner and Street, 1978), although this has been disputed (Dunwell, 1978). Presumably, the pollen dimorphism would reflect the different genetic backgrounds of the populations.

b) Effect of Activated Charcoal

The addition of activated charcoal to solid culture media has been reported to enhance plantlet or embryoid yields from cultured anthers of several species, such as tobacco (Anagnostakis, 1974; Wernicke and Kohlenback, 1975; Kohlenbach and Wernicke, 1978), potato (Sopory et al., 1978), and maize (Miao et al., 1978). Constantin et al. (1977) have provided evidence that activated charcoal inhibits callus growth and shoot development in pith explants of tobacco (cv. Wisconsin 38) by binding to the growth regulators added to the culture medium. Fridborg and Eriksson (1975) also proposed that substances, perhaps auxin, are removed from the medium by activated charcoal. Recently, Kohlenbach and Wernicke (1978)

concluded that the beneficial effects of activated charcoal result from the adsorption of inhibitors present in agar, rather than the removal of toxic substances originating from the tissues of the anther, such as the inhibitory wall factors postulated by Dunwell (1976).

The significant differences in the callusing frequencies found in cultures of L. angustissimus and L. tenuis between the regular and the charcoal-plus media may not be regarded as conclusive because of the small number of anthers cultured. However, if these differences are real, this may indicate that, if activated charcoal acts by binding to growth regulators present in the culture medium, the levels of endogenous hormones in the explants must be relatively low, which would explain the reduced callus formation. Callusing of anthers of several Lotus species was induced only on hormone-supplemented media (Niizeki, 1971). In the present studies, the inclusion of activated charcoal in the culture media did not promote pollen callusing or embryogenesis.

8. Physiological Status of Donor Plants

In Lotus it remains to be determined to what extent the physiological state of the donor plant influences the induction of pollen divisions. Indications to the effect that the age of the donor plant may play an important role have been obtained from studies on annual species (Sunderland, 1971; Dunwell, 1976). Similar studies with perennial species, such as species of Lotus, are lacking and need to be undertaken.

9. Cytology of the Regenerant

A few haploid cells (more correctly dihaploid cells, $2n=12$) were observed in root tips collected from a plant of L. corniculatus (B259) regenerated from anther culture. These cells were found among a mixed population composed of principally tetraploid cells and a smaller

proportion of octoploid cells. These findings may indicate that the regenerant originated from a callus which included cells with a haploid chromosome complement. These cells presumably were derived from microspores, although the unlikely event of somatic reduction cannot be ruled out entirely.

It has been suggested that plants regenerated from tissue culture derive from single cells and that cytochimeras may arise from chromosomal number changes that occur during in vitro growth (D'Amato, 1977). However, evidence has been presented for a multicellular provenance which, in the case of a mixoploid callus, would explain also the occurrence of cytochimeras (Sree Ramulu et al., 1976b; Mix et al., 1978). It was not possible to obtain details on the ploidy of the callus from which the Lotus plant differentiated since the callus was no longer available for investigation at the time the plant had matured. Even if an analysis of the callus could have been performed, it may not have been representative of the situation which exited at the time the plantlet differentiated. It is well known that ploidy levels evolve in prolonged culture (Guo, 1972; Sunderland, 1973; Niizeki, 1977; D'Amato, 1975, 1977, 1978). Also, since the cytological examinations were performed on the mature regenerant, it may be that the haploid cell populations had been eliminated almost entirely in the course of development as shown in cultures of haploid Pelargonium (Bennici, 1974) and in anther-derived barley plants (Foroughi-Wehr et al., 1976).

As discussed above, the Lotus regenerant may have arisen for a single cell or from a group of cells. In the event of a single cell origin, this cell would give rise to a genetically uniform plant in which

the cells would possess the same genotype. Since this plant arose from callus cells which have undergone many divisions during which time both chromosomal and gene mutations could occur, the regenerant could be phenotypically or genotypically different from the donor plant. If a multicellular origin was involved, it is possible that the regenerant could consist of a mosaic of cells also containing different chromosome numbers or gene combinations. Solving this type of puzzle may only be feasible with the aid of suitable markers.

B. Pollen Culture

The culture of isolated pollen grains offers several advantages over anther culture, as pointed out by a number of investigators (Sharp et al., 1972b; Debergh and Nitsch, 1973; Reinert et al., 1975; Laneri and Chirila, 1976). For instance, the technique facilitates the study of the various aspects of pollen embryogenesis and the determination of the origin of non-haploid plantlets, and eliminates the problem of somatic callusing.

An interesting phenomenon was observed in cultures of microspores of L. corniculatus (cv. Viking). Following the first pollen mitosis, Niizeki (1971) showed that the two nuclei moved to opposite poles and cytokinesis occurred. One of the nuclei divided a second time, and a three-celled pollen grain, in which the cells were linearly arranged, was produced. Niizeki (1977) concluded that "the cultured pollen grains had acquired some unknown factor which appears to be lacking when the anthers possessing the pollen grains were cultured. This factor is effective at least in the initiation of the multicellular condition, although it is not sufficiently effective for the continued development into a callus or an embryoid".

The negative results obtained in the present studies do not permit one to reach a definite conclusion as to the ability of Lotus pollen to undergo embryogenesis when cultured in the absence of the anther.

C. Pollen Tube Culture

The culture of pollen tubes of Lotus species permitted the observation of both branched and enlarged pollen tubes. In Lotus, the formation of callus tissue from the pollen tubes was not induced as has been reported in gymnosperms (Tulecke, 1953, 1957, 1959, 1960), nor was the initiation of embryoid development as observed in tobacco by Nitsch et al. (1968). The latter authors noted in their anther culture studies the formation of embryo-like structures at the extremity of the tubes of germinating grains. They observed that nuclei migrated towards the tip of the tubes and underwent divisions to form multicellular structures on which cotyledons differentiated.

In Lotus, the addition of the synthetic auxin 2,4-D to the culture medium may have induced branching of the pollen tubes, as reported in Lilium auratum Lindl. by Iwanami (1956). The sugar concentration of the germination medium has been involved also in the development of branched pollen tubes in the California poppy, Eschscholtzia californica Cham. (Beatty, 1943). Beatty (1943) also established a positive correlation between tube diameter and the sugar concentration used in the germination medium. Similarly in Lotus, it is possible that the high sucrose contents of the germination medium (≅ 10%) may have contributed to the formation of branched and enlarged pollen tubes.

D. Ovary and Ovule Culture

In Lotus, studies of cultures of unpollinated ovaries have not been reported previously and, in general, little information is available in the literature on the cytological behavior of in vitro-cultured unfertilized ovules (Sachar and Kapoor, 1958, 1959; Haccius, 1974; Lintilhac and Jensen, 1974).

1. Effect of the Culture Media on Callus Formation and Induction of Parthenogenesis

With the view of inducing callus formation from ovaries and ovules and the parthenogenetic development of female gametophytes, a wide range of Lotus germplasm was utilized. A comparison of the culture media in terms of their efficiency as callus promoters, or parthenogenetic inducers, becomes rather difficult, since different species and cultivars were assayed on different media and one cannot ignore the genotypic differences that certainly exist, as shown in anther culture studies.

In general, in Lotus, the tissues of the ovary may be induced quite easily to produce callus when placed under in vitro culture conditions. This is shown by the relatively high frequency of callusing observed in several species. Only in rare instances was callusing initiated on the ovules. The responsive ovules had been isolated on the culture medium, which seems to indicate that a direct contact with the culture medium was a prerequisite for callus induction.

The level of callusing was rather low in species cultured on the medium of Murashige and Skoog (Type I), whether or not the medium was supplemented with growth regulators. There was one exception, a single genotype of L. corniculatus which responded to a combination

of zeatin and IAA; ovules which were isolated on the medium produced callus from the integuments, but development of the gametophyte did not occur.

The species cultured on a Murashige and Skoog's medium in which the mineral composition had been reduced by half (Type III) responded with a very high level of callusing. This callusing was apparently independent of the type of growth regulators used (2,4-D and BAP), or the particular combinations of these substances, and in L. mascaensis, the frequency of callus induction was similar on the basal medium and the growth regulator-supplemented media. In the culture of unpollinated ovaries of Zea mays, Uchimiya et al. (1971) found that the frequency of callus formation was higher on MS medium than on Nitsch's medium (1951). The authors suggested that ovary callusing in maize was favored by high inorganic salt concentrations, such as is present in the Murashige and Skoog's medium.

Gamborg's medium was conducive to callus formation from Lotus ovaries, and in some cases, from the ovules of the different species cultured at various stages of development. MacDonald (1974) reported that Gamborg's medium best supported the growth of callus tissue from root cultures of L. corniculatus cv. Viking in comparison with the other media assayed, namely, MS, Miller's (1963), Liau and Boll's (1970), and Nagata and Takebe (1971). MacDonald (1974) found that the organic fraction of all media contributed less to differences in final fresh weights of callus than the salt composition of the media. The culture media with relatively low salt concentrations, that is, Gamborg's, Miller's, and Nagata and Takebe's, proved to be more suitable than the media with relatively high salt concentrations,

namely, MS, and Liau and Boll's media. MacDonald (1974) concluded that Gamborg's medium was the most suitable for the growth of Lotus cultures because of its specific calcium and nitrate concentration. These findings may confirm the high degree of callusing observed on Lotus ovaries cultured on Veliky and Martin's culture medium (1970). This medium contains also low nitrate and calcium levels.

The potato medium successfully utilized to improve embryoid and plantlet yields from cultured anthers (Anonymous, 1976; Wenzel et al., 1977a) did not have any promotive effect on the induction of parthenogenesis in Lotus, whether or not the medium was supplemented with growth regulators. Additional studies in Lotus with the potato medium are needed to assess its value.

2. Effect of Temperature Treatments

A few reports are present in the literature where an association between the production of haploid plants and the use of temperature treatments has been established or hypothesized (cold: Datura, Blakeslee et al., 1922; Crepis, Hollingshead, 1928; rye, Müntzing, 1937; heat: Triticum, Chizaki, 1934; rye, Nordenskiöld, 1939). However, an explanation of the mechanism by which temperature induces parthenogenetic development is still needed. In the present studies with Lotus, temperature treatments, either cold (4°C) or hot (30°C), did not promote parthenogenesis in the cultured ovules of L. corniculatus (cv. Leo) and L. emeroides. Perhaps more extensive trials with additional species may show a relationship.

3. Effect of High Sucrose Concentration

San Noeum (1976) observed that haploid plantlets were produced

from ovaries which had been cultured on a medium supplemented with high sucrose levels (8 to 12%), but not from those inoculated on the same medium supplemented with lesser amounts of sugar. In cultured fertilized ovules, it was determined that high sucrose concentrations created an osmotic pressure which favored the development of the embryos (Kameya and Hinata, 1970; Wakiżyka and Nakajima, 1974). It remains uncertain whether the promotive effects of high sucrose concentrations observed by San Noeum in barley were solely due to an influence on the osmotic pressure, or whether sucrose played a metabolic role. It was shown that the promotive effects of high sucrose levels (6 to 12%) in cultured anthers of certain cereals (Clapham, 1973; Ouyang et al., 1973; Picard and de Buyser, 1973; Chen, 1977; Miao et al., 1978) were not only osmotic but also metabolic (Miao et al, 1978). Thorpe (1978) concluded from his studies on shoot formation in callus cultures of tobacco that sucrose not only played an important role as a carbon source and an osmotic agent, but that it constituted a source of reducing power essential for the biosynthetic reactions occurring during organogenesis.

In Lotus, increasing the sucrose concentration of the culture medium to levels comparable to those used by San Noeum (1976) had no promotive effect on the induction of parthenogenesis.

4. Effect of Activated Charcoal

The possible roles of activated charcoal in culture media have been discussed previously (Section A-7-b). In Lotus, in all experiments where charcoal was included in the culture medium, no differences were found between charcoal-plus and charcoal-less media. If activated charcoal adsorbs growth regulators present in the culture medium, as

has been suggested by Fridborg and Eriksson (1975) and Constantin et al. (1977), then in Lotus species where callusing occurred on charcoal-plus media this may indicate that the levels of endogenous hormones in the ovaries must have been sufficient to induce the formation of a callus tissue. The reverse situation (i.e. no callus formation, low endogenous hormonal levels) would be also true. In Lotus, the presence of activated charcoal in the culture medium did not promote the induction of parthenogenesis in any of the various species cultured.

5. Effect of Agar

The reported observations that agar contains substances inhibitory to pollen embryogenesis (Section A-3-c) prompted such studies with Lotus ovary cultures. The callusing response of ovaries of L. corniculatus was similar on liquid and solid media, and ovule development did not occur in any of the treatments.

6. Cytological Examination of Callus-Derived Roots

Cells with haploid (2x) (more correctly, dihaploid) complements were observed along with tetraploid (4x) cells in the meristem of a root which had differentiated from a callus produced from a cultured ovary of L. emeroides (Results, Section D-3-a). The presence of these haploid cells implies that either they resulted by a phenomenon of somatic reduction that occurred in the tetraploid root, presumably of somatic origin, or that the root developed from a mixoploid callus containing haploid cells. In calluses and cell suspensions, it has been shown that roots may form from large multicellular aggregates (Thomas and Davey, 1975). These haploid callus cells may have originated from

the division of gametophytic cells (i.e. the embryo sac) or from the products of meiosis (i.e. the megaspores) since the ovules were cultured from the premeiotic to postmeiotic stages of development. Uchimiya et al. (1971) reported the presence of haploid cells in calluses derived from cultured ovaries of maize. However, only diploid cells were observed in roots derived from these calluses. Haploid cells were also found in calluses obtained from cultured ovules of eggplant.

The establishment of haploid cell lines may have been favored by the genotypic constitution of L. emeroides which is considered to be an autotetraploid (Larsen, 1960). A dihaploid condition in this species would, presumably, present a balanced situation.

E. In Vitro Culture of Inflorescences

The observations made in the course of these experiments indicated that culturing inflorescences, or flower buds, was not conducive to the formation of callus from the anthers or the ovaries, or to the induction of androgenesis or parthenogenesis.

However, calluses were formed when anthers of L. angustissimus were inoculated on medium subtypes 1 and 3 without charcoal (corresponding to medium subtypes 3 and 4 in Table VI, respectively) and submitted to the same culture conditions as the cultured inflorescences. The few ovaries of L. angustissimus, which were cultured on the same media and submitted to the same cultural conditions did not exhibit callus formation or internal development. In this Lotus species, calluses were produced only on anthers dissected on the culture medium. Sunderland and Dunwell (1971) tested the possibility that the floral parts exerted

an inhibitory influence on the embyogenic process in tobacco pollen. Their observations indicated that direct contact of the anthers and the culture medium was necessary to induce embryogenesis. It was inferred also from their studies that the somatic tissues other than those of the anther were not involved in the induction process. Inserting the flower buds vertically in the medium simply prevented the anthers from being in contact with the medium.

In the experiments with inflorescences of Lotus, the ovaries that belonged to the more mature florets which were cultured (petals unexpanded) continued their development. This seemed to indicate that they were receiving nutrients from the culture medium. However, parthenogenetic development of the egg or other cells of the embryo sac was not triggered and the ovules aborted. The younger buds were not induced to develop further and soon degenerated after the onset of the culture.

Preil et al. (1978) noted that the formation of callus on the ovaries and stigmas from cultured florets of Gerbera jamesonii occurred rarely. Callusing from the ovary or the stigmatic area was not observed in the cultured Lotus species. It would appear that callusing is only induced on the part of the explant that is in direct contact with the culture medium.

The formation of callus tissues and roots on the peduncles of cultured inflorescences, flowers, and developing ovaries, has been observed in several species (LaRue, 1942; Nitsch, 1963; Mizske and Skucińska, 1975) and has been correlated with the presence of auxin in the culture medium. The natural auxin indoleacetic acid as well as the synthetic auxins have been shown to be involved in root initiation

from cuttings and tissue cultures (Thimann, 1977). The fact that cuttings of Lotus species can be easily rooted with the use of commercial preparations of indolebutyric acid, a synthetic auxin, suggests that the pedicels may respond in a similar way when placed in a culture medium supplemented with auxins, such as medium subtype 3 (which contained 2,4-D and benazolin). Benazolin is known for its auxin-like properties in tissue cultures (Ingram and Butcher, 1972), although its precise mode of action is not understood.

F. Polyembryony

The search for polyembryonic seeds in Lotus led to the recovery of five sets of twins from two species, L. corniculatus and L. pedunculatus var. villosus.

Twin seedlings in diploid L. pedunculatus have not been reported previously. Since these seedlings degenerated shortly after their transfer to soil and did not reach the expanded cotyledon stage, it was impossible to determine their ploidy and obtain any clues as to their mode of origin.

The occurrence of twin seedlings in L. corniculatus was briefly mentioned by Skovsted (1939). The author, however, did not describe the seedlings, nor did he examine them cytologically.

In the present study, all recovered L. corniculatus twins were composed of unequally-sized members, a feature which suggested that the smaller member may have been haploid, as previously described in asparagus (Marks, 1973), soybean (Ahmad et al., 1975), and interspecific hybrids of Gossypium (Skovsted, 1939). However, the chromosome counts performed on three sets of Lotus twins established that both members of

each pair were tetraploid. Cases of dissimilar twin seedlings with identical chromosome numbers have also been reported, for example by Greenshields (1951) in alfalfa, and Skovsted (1939) in red clover, timothy, alsike clover, carrot, flax and Agrostis alba L. Skovsted (1939) concluded from his studies that it was impossible to assess the ploidy of the seedlings by their differences in size. These variations in size have been considered the result of competition for nutrients between the two embryos (Skovsted, 1939; Cameron, 1949).

In the course of this study, it was observed that polyembryonic seeds could not be selected on the basis of seed size. Likewise, twin seedlings were not found during an extensive screening for large seeds in L. corniculatus (W. F. Grant, Macdonald College, Ste-Anne de Bellevue (Que.), pers. comm.). Kenworthy et al. (1973) emphasized that, in soybean, twins "could not be determined by visual examination of dry mature seed". The authors also mentioned that there did not appear to be a correlation between seed weight and the presence of twin embryos.

It is interesting to note that, in L. corniculatus, all the twin sets were produced in one particular source of germplasm, namely, the cultivar Mirabel, but even in this cultivar with a rather low frequency (0.028%). No polyembryonic seeds were found in cultivar Leo, although a comparable number of seedlings to that of cultivar Mirabel were examined. This most likely reflects genotypic differences. Variations in the frequency of polyembryony between genotypes have been observed by Blank and Allison (1963) in Gossypium hirsutum and Ahmad et al. (1977) in soybean. It is rather difficult to make a valuable comparison between the other germplasm sources and cultivar Mirabel due to the much smaller number of seedlings screened.

Conjoined twins were not observed in any of the genotypes investigated. A few seedlings with bifurcating root tips were observed, but these seedlings possessed only one growing point. The origin of the L. corniculatus twins may only be speculated upon: 1) they may have arisen from twin zygotes, although the observation of double embryo sacs in ovules of L. corniculatus or, for that matter, in other Lotus species, has not been reported in the literature; 2) they may have originated from a single zygote which separated into two distinct embryos at an early stage of development; 3) one member may have arisen from the zygote and the other member from another somatic cell of the ovule; in this regard, nucellar embryony may be possible, but unlikely at fertilization time, since the nucellus has almost entirely disintegrated at this stage (Hansen, 1953); embryogenesis of the other somatic tissues has not been observed in the few embryological studies performed in this species (Souèges, 1929; Hansen, 1953; Jaranowski and Wojciechowska, 1963; Wojciechowska, 1963); 4) another possibility, as suggested by Maheshwari and Sachar (1963), is that a haploid (2x) cell of the embryo sac may have become tetraploid (4x) following endoduplication of the chromosomes and undergone embryogenesis simultaneously with the development of the zygote. Similarly, simultaneous fertilization of the egg cell and another cell of the embryo sac may lead to the production of twins, but whether these would develop in the absence of an endosperm is quite doubtful. This is illustrated by interspecific hybridization experiments in Lotus where the failure of endosperm development leads to the degeneration of the embryos (Jaranowski and Wojciechowska, 1963).

CONCLUSION AND SUMMARY

Various attempts were made to trigger androgenesis in several Lotus species. Supplementation of the four culture media [Murashige and Skoog (1962), Gamborg (1970), Veliky and Martin (1970), and a Potato medium] with several growth factors (CM, 2,4-D, IAA, NAA, BN, BAP, and K) resulted in additional nuclear and cellular divisions in the pollen in the following cases: L. emeroides anthers cultured on liquid MS (Type III) supplemented with BAP alone or BAP + 2,4-D; L. villosus anthers cultured on the potato medium supplemented with K and 2,4-D; and possibly, L. corniculatus anthers cultured on MS (Type III) supplemented with BAP and IAA as indicated by the presence of haploid (2x) cells in root tips of a regenerated plant. High sucrose levels did not promote androgenesis in L. alpinus pollen grains, nor did the addition of activated charcoal to different culture media in any of the species assayed. Somatic callusing appeared to be favored by the use of a medium containing low levels of nitrate and calcium. Genotypic differences were observed in the callusing response for several species.

From the paucity of positive results obtained, it is considered that the media utilized were not ideal for inducing haploidy in Lotus and that further research should be carried out in order to improve or design a culture medium better fitted to the particular requirements of cultured anthers of Lotus species.

Pollen and pollen tube cultures were not successful in inducing additional divisions and formation of calluses in any of the species

cultured.

Ovary and ovule culture of Lotus species attempted on four different culture media [Murashige and Skoog (1962), Gamborg (1970), Veliky and Martin (1970), and a Potato medium] supplemented with different growth factors (CM, 2,4-D, NAA, NlAA, α,β-D, IAA, BN, BAP, K, and Z) possibly induced parthenogenetic development in L. emeroides cultured on liquid MS (Type III) supplemented with 2,4-D and BAP, since haploid (2x) cells and aneuploid cells with somatic chromosome numbers less than 28 were observed in a root produced on an ovary-derived callus cell line. High sucrose concentrations did not promote the induction of parthenogenesis from the cultured ovaries and ovules, nor did the presence of activated charcoal in the culture medium in the various species assayed. As shown in the anther culture studies, somatic callusing appeared to be favored by the use of media containing low concentrations of nitrate and calcium. Except for the case mentioned above, ovary and ovule culture failed to induce parthenogenesis in Lotus. Further research is needed to investigate the cytological behavior of cultured unfertilized ovules of Lotus. It is suggested that by selecting an appropriate culture medium it should be possible to induce the formation of callus tissues and the differentiation of haploid plants from female gametophytes of Lotus species.

In Lotus, the culture of inflorescences does not appear to favor somatic callusing from anthers and ovaries, or to promote pollen embryogenesis and ovule parthenogenetic development. This technique may be of value in the study of adventitious root formation.

Screening for polyembryonic seeds in Lotus led to the recovery of five sets of twins from two species, namely, L. pedunculatus var.

villosus and L. corniculatus cv. Mirabel. The twin seedlings were all tetraploid. An improved method for raising the twin seedlings to maturity remains to be devised and for this purpose, hydroponic techniques may prove useful. Since a number of twin seedlings were found in L. corniculatus cv. Mirabel, it is considered that further investigations of this cultivar for the recovery of haploid seedlings may be rewarding.

APPENDIX 1

Murashige and Skoog's culture medium (1962)

Compound	mg/l
KNO_3	1900
NH_4NO_3	1650
KH_2PO_4	170
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
$MnSO_4.4H_2O$	22.3
H_3BO_3	6.2
$ZnSO_4$	8.6
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Na_2EDTA*	37.3
$Fe_2SO_4.7H_2O$	27.8
Glycine	2.0
Thiamine.HCl	0.1
Nicotinic acid	0.5
Pyridoxine.HCl	0.5
myo-Inositol	100.0

*Disodium ethylenediaminetetraacetic acid

APPENDIX 2

Gamborg's culture medium (1970)

Compound	mg/l
KNO_3	2500
$(NH_4)_2SO_4$	134
$NaH_2PO_4.H_2O$	150
$CaCl_2.2H_2O$	150
$MgSO_4.7H_2O$	250
$MnSO_4.H_2O$	10.0
H_3BO_3	3.0
$ZnSO_4.7H_2O$	2.0
KI	0.75
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
FeDPTA*	28.0
Thiamine.HCl	10.0
Nicotinic acid	1.0
Pyridoxine.HCl	1.0
myo-Inositol	100.0

*Iron chelate of diethylenetriaminepentaacetic acid, obtained from Geigy Agricultural Chemicals, Ardsley, New York, U.S.A.

APPENDIX 3

Velitky and Martin's culture medium (1970)

Compound	mg/l
NaH_2PO_4	150
Na_2HPO_4	20
KCl	200
$MgSO_4.7H_2O$	250
$(NH_4)_2SO_4$	100
KNO_3	800
$CaCl_2.2H_2O$	200
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.25
$MnSO_4.H_2O$	4.0
$ZnSO_4$	1.5
$CoCl_2.6H_2O$	0.25
H_3BO_3	5.0
KI	0.05
$FeSO_4.7H_2O$	13.9
Na_2EDTA*	18.6
Casein hydrolysate	2000
Thiamine.HCl	0.5
Pyridoxine.HCl	0.5
Nicotinic acid	1.25
Calcium panthotenate	1.0
myo-Inositol	100.0

*Disodium ethylenediaminetetraacetic acid

APPENDIX 4

Potato culture medium

Compound	Concentration
Potato extract	20% (w/v)
FeNaEDTA*	40 mg/l
Sucrose	9% (w/v)

*Sodium ferric ethylenediaminetetraacetic acid

APPENDIX 5

Gamborg's B5 (1968)-soybean culture medium

Compound	Concentration
KNO_3	2500 mg/l
$CaCl_2.2H_2O$	150
$NaH_2PO_4.H_2O$	150
$(NH_4)_2SO_4$	134
$MgSO_4.7H_2O$	250
$MnSO_4.H_2O$	10.0
H_3BO_3	3.0
$ZnSO_4.7H_2O$	2.0
KI	0.75
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6.H_2O$	0.025
Na_2EDTA*	37.3
$FeSO_4.7H_2O$	27.8
Soybean extract	10% (w/v)
Sucrose	12% (w/v)

*Disodium ethylenediaminetetraacetic-acid

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Figure 1. Anthers cultured on solid medium.

2. Combined anther and ovary culture on solid medium.

3. Combined anther and ovary culture on liquid medium.

4. Ovaries cultured on solid medium supplemented with (left) or without (right) activated charcoal.

5. Inflorescences cultured on solid medium supplemented with (left) or without (right) activated charcoal.

6. Inflorescences cultured on liquid culture medium.

7. Germinating pollen grains from anther culture of L. alpinus (4x) on MS (Type II) supplemented with 8% sucrose without activated charcoal, ca. X 250.

8. Callusing anthers of L. mascaensis cultured on liquid MS (Type III), subtype 3.

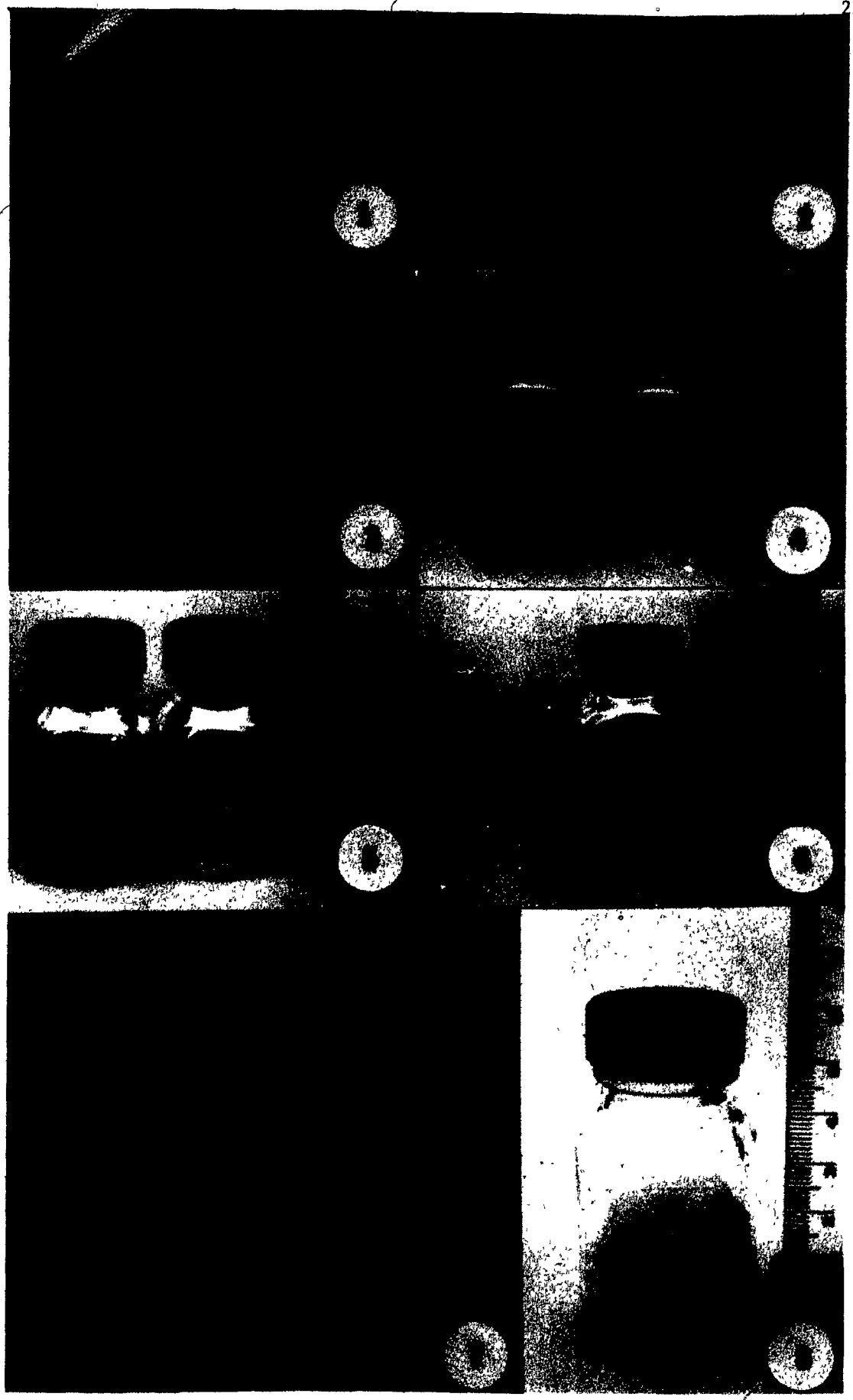

Figure 9. Callusing anthers of L. emeroides cultured on liquid MS (Type III), subtype 3.

10. Root formation from anther-derived callus tissue of L. emeroides subcultured on the differentiation medium.

11. Plantlet formation from anther-derived callus tissue of L. emeroides subcultured on the differentiation medium.

12. Shoot regeneration from anther-derfved callus tissue of L. emeroides subcultured on the differentiation medium.

13. Multicellular-like pollen grain observed in anther-derived callus tissue of L. emeroides cultured on liquid MS (Type III), subtype 4. ca. X 1360.

14. Multicellular pollen grain-like structure observed in anther-derived callus tissue of L. emeroides cultured on liquid MS (Type III), subtype 3, with a cold pretreatment. phase contrast, ca. X 910.

15. Heart-shaped embryo-like structure from a cultured anther of L. corniculatus on MS (Type III), subtype 6. ca. X 140.

16. Shoot formation of L. corniculatus from anther-derived callus tissue [MS (Type III), subtype 7] subcultured on the differentiation medium.

17. L. corniculatus donor plant.

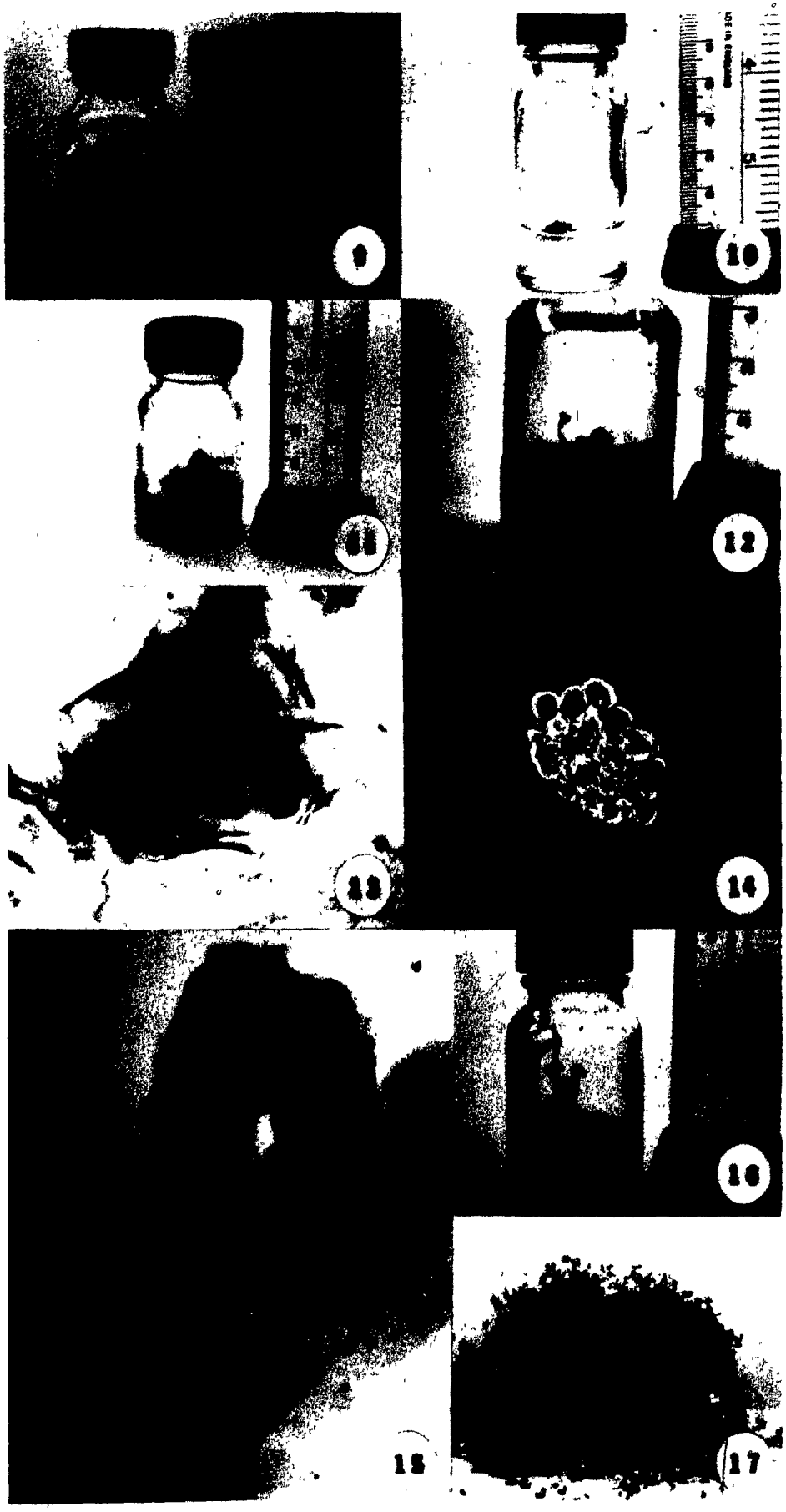
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Figure 18. *L. corniculatus* plant regenerated from anther culture on MS (Type III), subtype 7.

19. *L. corniculatus* donor plant (left) and regenerant (right).

20. *L. corniculatus* regenerant. Side view of florets showing the complete enclosure of the keel by the wings.

21. *L. corniculatus* donor plant. Side view of floret showing the partial enclosure of the keel by the wings.

22. *L. corniculatus* regenerant. Calyx of floret with sparse pubescence.

23. *L. corniculatus* donor plant. Calyx of floret with dense pubescence.

24. *L. corniculatus* regenerant. Leaflets with ciliate margins.

25. *L. corniculatus* donor plant. Leaflets with smooth margins.

26. Meiosis in pollen mother cells of the *L. corniculatus* regenerant. Anaphase I cell showing three bivalents left at the equatorial plate. Phase contrast, ca. X 1360.

27. Meiosis in pollen mother cells of the *L. corniculatus* regenerant. Anaphase I cell showing two bivalents left at the equatorial plate. Phase contrast, ca. X 1100.

Figure 28. Meiosis in pollen mother cells of the L. corniculatus regenerant. Micronucleus (arrow) in quartets. Phase contrast, ca. X 640.

29. Mitosis in root tips of the L. corniculatus regenerant. Cell with 12 chromosomes (arrow). Phase contrast, ca. X 590.

30. Mitosis in root tips of the L. corniculatus regenerant. Cell with 24 chromosomes (one out of focus). Phase contrast, ca. X 1400.

31. Mitosis in root tips of the L. corniculatus regenerant. Cell with 48 chromosomes. Phase contrast, ca. X 1470.

32. L. villosus anthers cultured on the potato medium, subtype 2. Binucleate microspore showing symmetrical nuclei. ca. X 2430.

33. L. villosus anthers cultured on the potato medium, subtype 2. Binucleate microspore showing asymmetrical nuclei. ca. X 2680.

34. L. villosus mature pollen grain showing a round vegetative nucleus and a crescent-shaped generative nucleus. ca. X 2560.

35. L. villosus anthers cultured on the potato medium, subtype 2. Trinucleate pollen grain (arrow) composed of two small, darkly staining nuclei, and one larger, more lightly staining nucleus. ca. X 760.

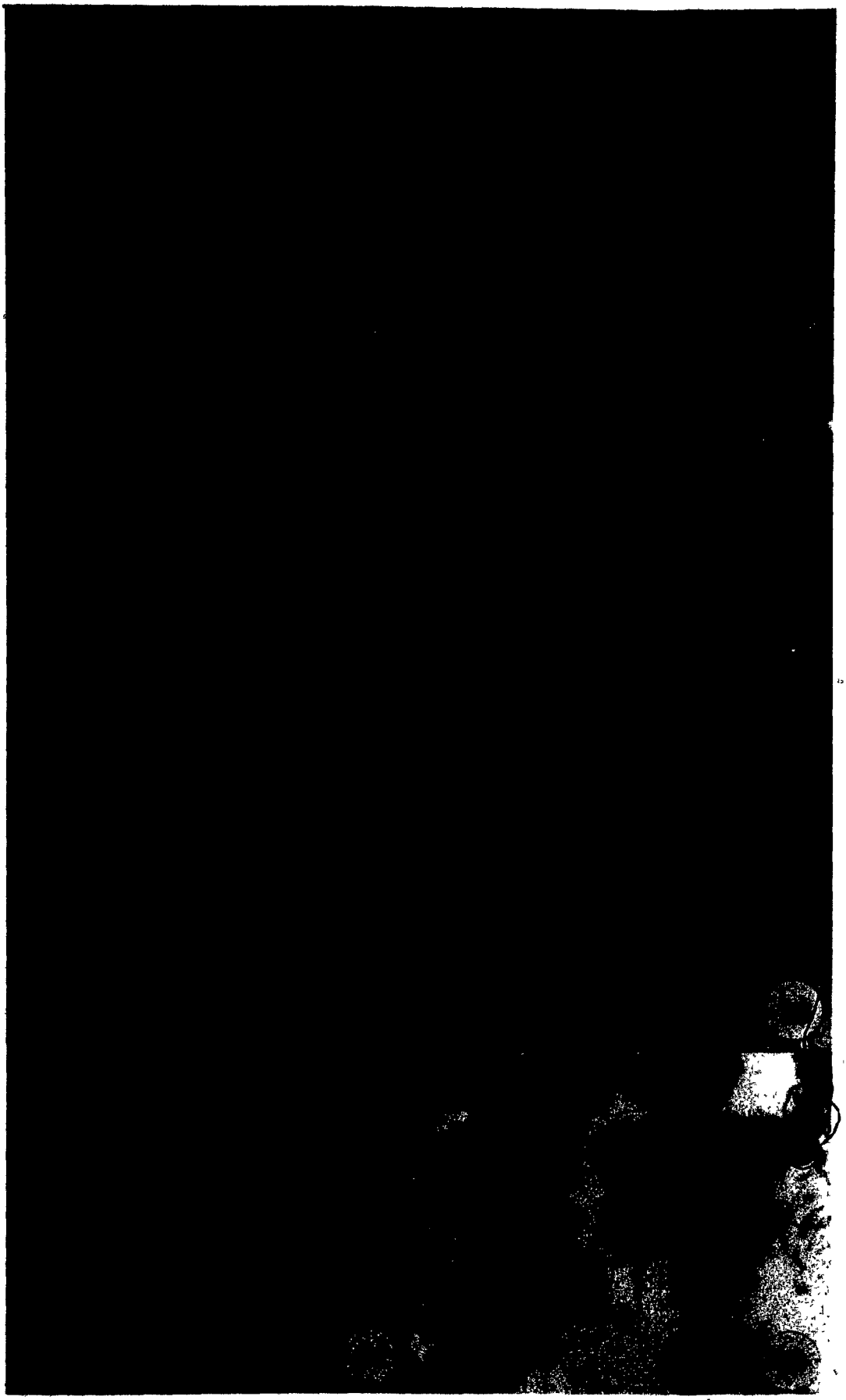


Figure 36. *L. villosus* anthers cultured on the potato medium, subtype 2. Tetranucleate pollen grain. ca. X 1580.

37. *L. villosus* anthers cultured on the potato medium, subtype 2. Multicellular pollen grain. ca. X 1400.

38. *L. villosus* anthers cultured on the potato medium, subtype 2. Multicellular pollen grain and non-induced microspore. ca. X 1360.

39. *L. emeroides* pollen cultured on the B5-soybean medium supplemented with 2,4-D. Mature binucleate pollen grains. ca. X 760.

40. *L. corniculatus* pollen tube culture. Branching pollen tube. Phase contrast, ca. 590.

41. *L. corniculatus* pollen tube culture. Enlarged pollen tube. Phase contrast, ca. X 540.

42. Ovary-derived callus tissue of *L. corniculatus* [MS (Type III), subtype 7] subcultured on the differentiation medium.

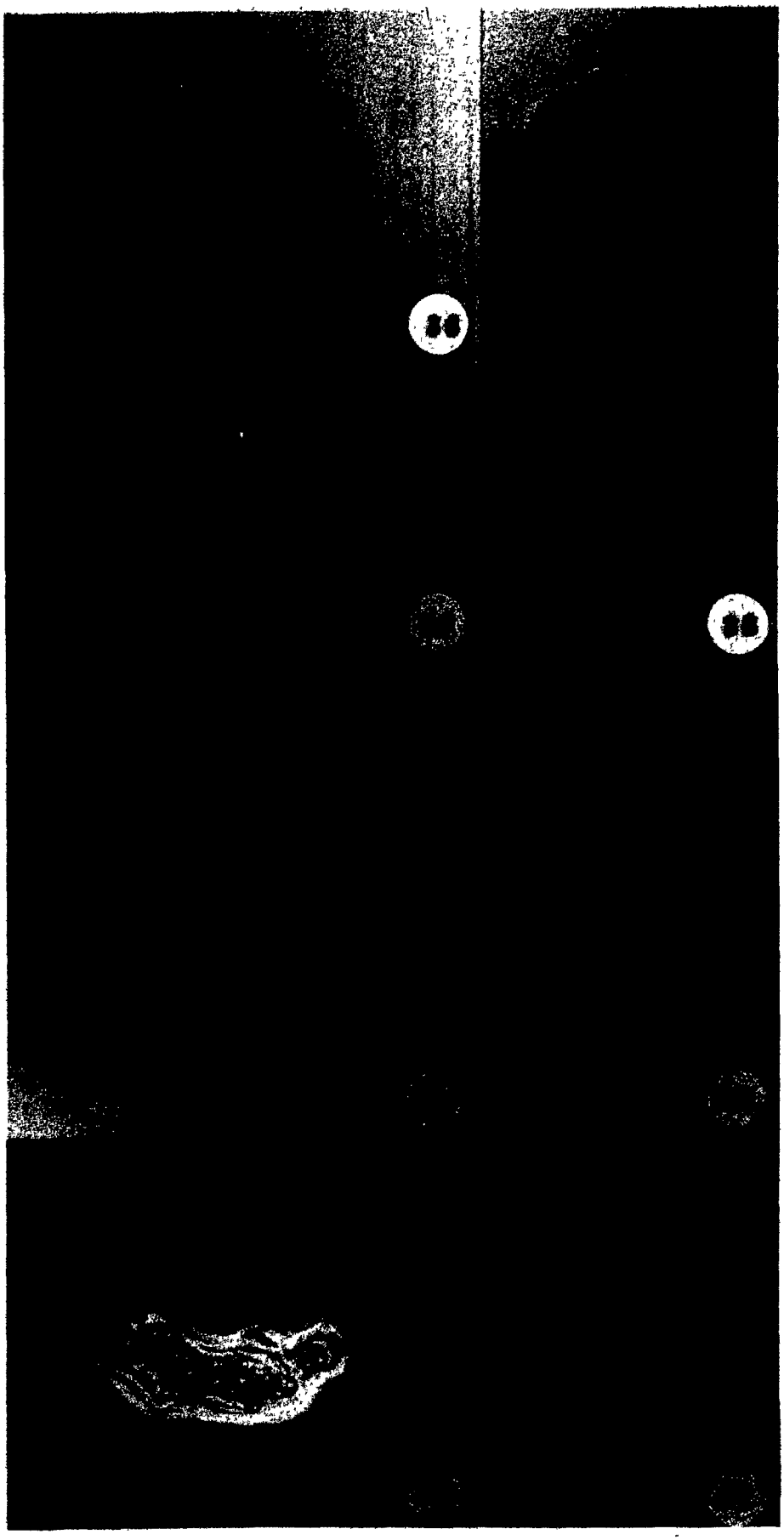


Figure 43. Shoot formation of L. corniculatus from ovary-derived callus tissue [MS (Type III), subtype 7] subcultured on the differentiation medium.

44. Callusing ovaries of L. mascaensis cultured on liquid MS (Type III), subtype 4.

45. Callusing ovaries of L. emeroides cultured on liquid MS (Type III), subtype 4.

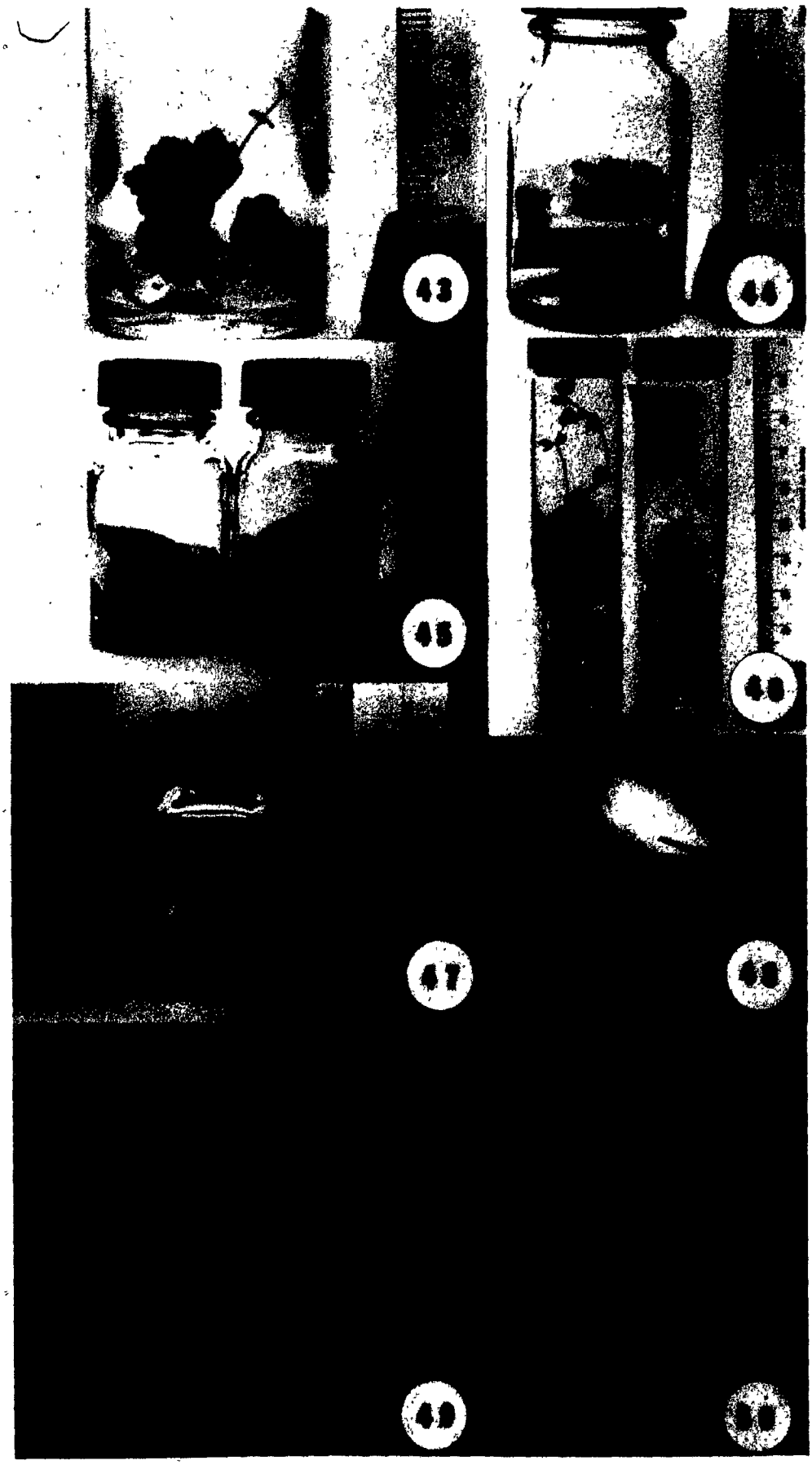
46. Ovary culture of (L. japonicus X L. alpinus)^2 on Gamborg's medium, subtype 4. Plantlet formation on ovary-derived callus tissue subcultured on the differentiation medium.

47. Degenerating flower buds from inflorescence culture of L. angustissimus on medium subtype 1, treatment a.

48. Twin seedling of L. corniculatus cv. Mirabel. ca. X 2.5.

49. Twin seedling of L. corniculatus cv. Mirabel, root tip. Cell with 24 chromosomes (one chromosome out of focus). Phase contrast, ca. X 1070.

50. Twin seedling of L. corniculatus cv. Mirabel, root tip. Cell with ca. 48 chromosomes. Phase contrast, ca. X 800.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