FUNADACION PARA LA INNOVACION AGRARIA
SRA MARGARITA D'ETIGNY
AV. SANTA MARÍA 2120
SANTIAGO

De mi consideración,

Me es grato enviar los informes Técnico y Financiero de la propuesta cod. FIA-FP-V-2004-1-A-034 realizada en el mes de febrero 2005.

Sin otro particular saluda atentamente,

Anja George

Osorno, 25 de mayo 2005

OFICINA DE PARTES - FIA
RECEPCIONADO
Fecha 6-6-05
Hora 16:50
Nº Ingreso 1108

AD

GOBIERNO DE CHILE
FUNDACIÓN PARA LA INNOVACIÓN AGRARIA

CONTENIDO DEL INFORME TÉCNICO Y DE DIFUSIÓN

Fecha de entrega del Informe
06 de junio de 2005
Nombre del coordinador de la ejecución
Anja George
Firma del Coordinador de la Ejecución

1. ANTECEDENTES GENERALES DE LA PROPUESTA
Nombre de la propuesta
Entrenamiento Practico en Biotecnología
Código
FIA-FP-V-2004-1-A-034
Postulante o Postulantes
Anja George
Entidad Patrocinante o Responsable
Anja George
Lugar de Formación (País, Región, Ciudad, Localidad)
Israel, Rehovot
Tipo o Modalidad de Formación (curso, pasantía, seminario, entre otros)
Entrenamiento
Fecha de realización (Inicio y término)
04 de febrero 2005 al 06 de marzo 2005

2. ALCANCES Y LOGROS DE LA PROPUESTA

Justificación y objetivos planteados inicialmente en la propuesta

El cultivo in vitro de plantas es una herramienta básica y necesaria en el desarrollo de estrategias comerciales y masivas. Dentro de este rubro se considera la micropropagación en diferentes tipos de medios de cultivo, como sólidos, líquidos y semi-sólidos, para diferentes especies.

Las especies vegetales se comportan diferentes entre una y otra, ya que se puede trabajar con especies bulbosas, herbáceas, leñosas y otras, y por ello se requiere investigar el comportamiento para determinar el desarrollo in vitro de las especies de interés, que en nuestro caso son los helechos.

Hoy hay entidades que trabajan con tecnologías muy avanzadas, que incluyen técnicas para acelerar el crecimiento y el desarrollo de las plantas, estableciendo de esta manera la forma optima de cultivo por cada especie.

Es necesario acercarse a estas entidades en forma práctica para visualizar y practicar estos avances y luego adaptarlos a la realidad productiva en nuestro país, o en este caso a la realidad del vivero.

En la actualidad, Israel es uno de los paises más avanzados en cuanto al desarrollo de la Biotecnología y su aplicación en la agricultura. Generalmente los países son muy celosos con la transferencia tecnológica hacia otros países. Es por ello que no se deber despreciar la oportunidad ofrecida por Investigadores de la Universidad Hebrea de Jerusalén para participar en un entrenamiento práctico y personalizado para transferir sus conocimientos en los avances de la Biotecnología. Conocimientos que hasta la fecha no están publicados y por ende solo se pueden obtener durante un entrenamiento práctico como el de esta propuesta.

El principal interés es obtener conocimientos en el desarrollo de medios líquidos; el trabajo con luz difusa; el uso de bioreactores; el establecimiento ex vitro de vegetales y otros temas que se puedan presentar con relación a la Biotecnología.

En cuanto al postulante se puede afirmar que Anja George lleva 6 años trabajando en el Vivero Río Tijeral en la propagación de helechos nativos con fines comerciales. Esta iniciativa fue apoyada inicialmente por FIA con el proyecto denominado "Tecnología y Desarrollo en la Producción Comercial de Helechos Nativos." Como resultado importante de la investigación se determinó que la propagación de helechos requiere de una tecnología más avanzada que la

usada comúnmente en un vivero comercial. En consecuencia de esto, Anja George postuló y luego se adjudicó un proyecto Fontec llamado: "Desarrollo de una Tecnología para propagar y Cultivar Especies de Helechos Nativos in vitro Destinados a la Exportación". Este nuevo proyecto se encuentra en su fase inicial, con la construcción de un laboratorio para el cultivo de tejido, especializado para la propagación de helechos en medios líquidos.

El entrenamiento en la Universidad Hebrea de Jerusalén es de suma importancia para permitir un avance significativo en el trabajo propuesto para el laboratorio, ya que promete capacitación en el uso de medios líquidos, que actualmente son usados para la propagación de helechos y muchas otras especies más.
Después de una profunda revisión de bibliografía y de Internet se pudo constatar que la información publicada con respecto a ese tema no esta completa, y se maneja entre los investigadores en forma muy reservada.

Además de lo anteriormente mencionado, permitirá establecer un contacto muy importante con este centro de investigación para futuras investigaciones o intercambios. Este interés lo manifestó la Sra. Rina Kamenetsky que va a trabajar como tutora acompañando a la postulante durante el entrenamiento. Cabe señalar que ella fue quien ofreció este entrenamiento a la interesada y la contactó con la Prof. Meira Ziv, del RH Smith Institute of Plant Science.

Laboratorios para el cultivo in vitro existen varios a nivel nacional, y por ello es importante contar con tecnología de punta para lograr un real avance tanto del punto de vista profesional como también desde el punto de vista comercial, ya que los conocimientos son un patrimonio muy difícil de adquirir y muy valioso dentro de una empresa, ya que permite marcar la diferencia entre una empresa y otra.
Anja George previo a la planificación de este entrenamiento ha visitado varios laboratorios de la Región, tanto Universidades como laboratorios privados y ahí se pudo constatar que en ninguna parte se pueden obtener informaciones completas y seguras para mejorar las tecnologías en el laboratorio. Las empresas y Universidades chilenas son aún más celosos con sus conocimientos y no están dispuestos a compartirlos con empresarios locales, además que no cuentan con una tecnología de punta en muchos casos y que esté probada en el cultivo de

helechos. Actualmente las Universidades consultan en el vivero Río Tijeral las experiencias obtenidas durante el proyecto FIA, ya que es el único estudio mas completo que se ha realizado en la propagación de helechos nativos hasta la fecha. Es decir, no se puede esperar mucho aporte desde una Universidad en Chile en el tema de la propagación de helechos en medios líquidos ya que no se está realizando. Es por eso que se tomó la decisión de participar en un entrenamiento en el extranjero que ofrece ventajas desde el punto de la tecnología utilizada y de la disponibilidad de los entrenadores para capacitar en forma gratuita a la postulante.

Objetivos alcanzados tras la realización de la propuesta

Participar en un entrenamiento practico para conocer avances tecnológicos en cultivos líquidos

Mejorar los conocimientos actuales

Participar en una investigación usando tecnología de punta

Transferir los conocimientos obtenidos durante el entrenamiento

Establecer contactos importantes con investigadores israelitas

Resultados e impactos esperados inicialmente en la propuesta

1. aumentar productividad del vivero
2. definir un protocolo para reproducir helechos nativos in vitro
3. confeccionar bioreactores para la multiplicación masiva de helechos
4. crear un contacto con Investigadores para apoyar el trabajo en el vivero desde afuera.

Resultados alcanzados

Durante el entrenamiento se lograron obtener conocimientos que ayudaron en forma significativa en el avance del proyecto que se esta llevando a cabo en el vivero. El programa de entrenamiento fue elaborado para comenzar con conocimientos básicos y luego finalizar con la introducción a tecnologías mas avanzadas. Como una parte del entrenamiento fue realizado en un instituto de investigación dentro de un programa de investigación se pudo además tener acceso a información reciente en temas como bioreactores, producción de especies bulbosas, peonías y otras.

En este momento se están aplicando las tecnologías conocidas en Israel, por lo tanto aún no se ven los resultados en el trabajo del laboratorio. Estos resultados son a mas largo plazo y

podrán recién verse en al menos 6-12 meses.

Resultados adicionales

Durante la estadía en Israel tuve la posibilidad de acompañar a la Sra. Rina Kamenetzky en dos oportunidades a terreno para asesorar productores de peonías en el desierto de Ber' Sheva y en Los Golan Hights. Gracias a estas visitas pude apreciar en terreno la producción forzada de peonías y establecer contactos con productores israelitas.

Aplicabilidad

El entrenamiento realizado en Israel fue muy puntual para conocer las posibilidades de reproducir helechos nativos chilenos in vitro, pero actualmente no se esta realizando la reproducción in vitro en otros viveros del país, por lo cual no es posible comparar la situación. Inicialmente se seguirá trabajando en el vivero Río Tijeral para finalmente establecer un protocolo de reproducción para distintas especies de helechos nativos. Este ES un proceso que durará algún tiempo, por lo tanto no es posible concluir algo en forma definitiva en este momento.

Detección de nuevas oportunidades y aspectos que quedan por abordar

La actividad realizada deja muy en claro que se debe seguir trabajando cambiando algunos aspectos técnicos anteriormente implementados y sobre todo utilizar la ayuda ofrecida por Meira Ziv y Rina Kamenetzky para resolver los problemas que se presenten durante la realización de la investigación.

3. ASPECTOS RELACIONADOS CON LA ORGANIZACIÓN Y EJECUCIÓN DE LA PROPUESTA

Programa de actividades

Fecha	Actividad	Objetivo	Lugar
07 al 12 de febrero 2005	Entrenamiento en técnicas básicas de uso de medios liquidos	**Conocer la tecnología aplicada a través del trabajo en el laboratorio**	RH Smith Institute of Plant Science
1 4 al 24 de febrero 2005	Introducción en el uso de Bioreactores	**Usar y adaptar Bioreactores para la siembra de esporas de helechos nativos**	RH Smith Institute of Plant Science
25 de febrero al 02 de marzo de 2005	de tejido de plantas y sus aspectos fisiológicos	**Aprender la tecnología aplicada para el cultivo masivo de especies vegetales in vitro**	RH Smith Institute of Plant Science

Al comparar las actividades programadas en la propuesta aprobada con las actividades que realmente se realizaron, cuando corresponda, señalar las razones por las cuales algunas de las actividades programadas no se realizaron como estaba previsto o se modificaron.

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Incorporar en este punto fotografías relevantes que contribuyan a describir las actividades realizadas.

Resumen Actividades realizadas:

06/02/05 Llegada a Tel Aviv a las 21:30 hrs.. Rina Kamenetzky me recibe y me lleva a la Reisfeld Residence donde está arrendado un departamento por un mes.
07/02/05 A las 10:00hrs. Encuentro con Meira Ziv para mostrarme el Campus, el laboratorio, personas con las cuales trabajaré y otros aspectos relevantes como la Biblioteca, Fotocopiadora etc.
Este día me entrega bibliografía para revisar y nivelar mis conocimientos (ver lista adjunta)
08/02/05 Se comienza con la preparación de medios para la propagación de helechos, tanto para medios líquidos como para sólidos.
09/02/05 Transferencia de helechos comprados a tubos de ensayos y botellas con medio líquido. Especies: Nephrolepis exaltata Boston Fern. Todo el día se trata de enseñar metodología para trabajar en forma aséptica en las cámaras de flujo laminar y en especial con helechos.
10/02/05 Salida a terreno con Rina Kamenetzky para ver producción de Peonías en el desierto de Be'er Sheva. Se visitaron en total tres productores en compañía de investigadores, coordinadores del proyecto y productores de todo el país.
13/02/05 SE preparan nuevos medios y se comienza a trabajar la introducción de material vegetal.
14/02/05 Sigue el trabajo en la cámara de flujo laminar con la transferencia de helechos. Sigue una profunda revisión de bibliografía.
15/02/05 Se trabaja con distintos medios para Canna indica, lavado de rizomas y su desinfección.
16/02/05 Este día se trata de la aislamiento de meristemas bajo la tutoría de Vered. Se realizan cortes con el ejemplo de Canna indica y helechos como Nephrolepis. El trabajo contempla identificación de meristemas vegetativos y aquellos que dan origen a flores como su aislamiento y ubicación en los tubos de ensayo. SE concuerda que no es fácil ver un meristema de helechos.
17/02/05 Se trabaja principalmente con la introducción de material vegetal.
20/02/05 Se debe realizar una transferencia de plantas que se han dividido anteriormente, ya que el medio líquido presenta muchas impurezas.
21/02/05 Salida a terreno en el sector de los Golan Hights. Se visitan dos productores y un centro de investigación en el pueblo de Nov. En este centro realizan importantes investigaciones en la producción de Proteas!
22/02/05 Primer día de trabajo en el Volcán Centre. Comenzamos con la preparación de medios para transferir Narcissus. Se enseña la propagación in vitro de esta especie a través de escamas y vástagos florales.
23/02/05 Salida a terreno para visitar un productor de una nueva especie para flores de corte Ornitogalum dubium. En este vivero están construyendo un laboratorio para reproducir distintas especies para la producción de flores de corte.
En la tarde trabajo en el Volcán Centre en la cámara de flujo laminar.
24/02/05 Sigue en trabajo en el Volcán Centre en la cámara de flujo laminar, además de ser introducida en el uso de distintos equipos de este laboratorio.
28/02/05 Salida a terreno con Rina Kamenetzky para visitar al productor de O. Dubium mas grande del país. En la tarde introducción en el uso de Bioreactores con Meira Ziv.
01/03/05 Visita la faria Agro Mashov en Tel Aviv.
02/03/05 Viaje de regreso.

15/04/05 Charla de difusión en la Reunión de Productores de Peonias
00/04/05 Charla de difusión en el liceo People Help People

Contactos Establecidos

Presentar los antecedentes de los contactos establecidos durante el desarrollo de la propuesta (profesionales, investigadores, empresas, etc.), de acuerdo al siguiente cuadro

Institución/ Empresa/Organi-zación	Persona de Contacto	Cargo	Fono/Fax	Dirección	E-mail
Universidad Hebrea de Jerusalem	Meira Ziv	Profesora	0528604 534		
Northern Research and Development Station	Menashe Cohen	Investigador		Nov	Menashe @migal.o rg.il
Vivero Bulbos	Elik Frank	dueño	0544743 005	-	frankelik @yahoo. com
Volcán Centre Bet Dagan	Rina Kamenetzky	Jefe dep. Horticola	0396835 11		rkamente @uoguel ph.ca

Tipo de Material	Nº Correlativo (si es necesario)	Caracterización (titulo)
Artículo	1	In vitro acclimatization
	2	Quality of microporopagated plants
	3	Background Knowledge
	4	Control of Oxidative Browning
	5	Mass propagation of fems through tissue culture

Foto	-	
Libro	-	
Diapositiva	-	
CD	1	Entrenamiento Israel (Fotos, documentos, informe)

4. PROGRAMA DE DIFUSIÓN EJECUTADO

Se realizaron dos charlas de difusión:

1) Reunión anual de productores de peonías: Fecha: 15 de abril de 2005. Se hizo una presentación con Data show a base de fotografías con especial hincapié en la producción forzada de peonías en Israel. Se tuvo que realizar este cambio de temática, ya que la producción in vitro de helechos no era de un interés para los asistentes. Se agrega archivo de Power Point en CD adjunta.

2) Liceo agrícola People Help People: Fecha 19 de abril de 2005. Al igual que en la reunión de productores se hizo una charla con Data Show mostrando fotos y explicando verbalmente. En este caso se sostuvo una reunión de aproximadamente una hora, donde los alumnos preguntaron todo lo relacionado a la charla y los programas FIA.

Material entregado en las actividades de difusión

Tipo de material	Nombre o identificación	Preparado por	Cantidad
Datashow	Entrenamiento en Biotecnología	Anja George	1

Participantes en actividades de difusión

Nombre	Ver listas en los anexos.

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Apellido Paterno	
Apellido Materno	
RUT Personal	
Dirección, Comuna y Región	
Fono y Fax	
E-mail	
Nombre de la organización, empresa o institución donde trabaja / Nombre del predio o de la sociedad en caso de ser productor	
RUT de la organización, empresa o institución donde trabaja / RUT de la sociedad agrícola o predio en caso de ser agricultor	
Cargo o actividad que desarrolla	
Rubro, área o sector a la cual se vincula o en la que trabaja	

Evaluación de las actividades de difusión

Las dos charlas que se realizaron tuvieron buena aceptación por parte de los asistentes, manifestándose esto en la gran cantidad de consultas que se hicieron. En cuanto a la publicación en el Diario Austral no se tuvo un buen resultado ya que hasta la fecha de entrega del informe no fue posible lograr que se publique un articulo. A pesar de reiteradas llamadas telefónicas la persona encargada no realizó la entrevista ya que no se pudo concretar una cita.

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5. EVALUACIÓN DE LA PROPUESTA

Organización durante la actividad (indicar con cruces)[1]

Ítem	Bueno	Regular	Malo
Recepción en país o región de destino según lo programado	X		
Cumplimiento de reserva en hoteles	X		
Cumplimiento del programa y horarios según lo establecido por la entidad organizadora	X		
Facilidad en el acceso al transporte	X		
Estimación de los costos programados para toda la actividad	X		

Evaluación de la actividad de formación

En esta sección se debe evaluar la actividad en relación a los siguientes aspectos:

a) Efectividad de la convocatoria
NO HUBO
b) Grado de participación de los asistentes (interés, nivel de consultas, dudas, etc)
FUE UN ENTRENAMIENTO PERSONAL
c) Nivel de conocimientos adquiridos en función de lo esperado (se debe indicar si la actividad contaba con algún mecanismo para medir este punto)
NO SE REALIZÓ
d) Calidad de material recibido durante la actividad de formación
MUY BUENO, YA QUE SE REALIZARON EN FORMA PRACITA LAS INSTRUCCIONES.

[1] En caso de existir un ítem Malo o Regular, señalar los problemas enfrentados durante el desarrollo de la actividad de formación, la forma como fueron abordados y las sugerencias que puedan aportar a mejorar.

e) Nivel de adecuación y facilidad de acceso a infraestructura/equipamiento necesario para el logro de los objetivos de la actividad de formación.
EXCELENTE
f) Indique las materias que fueron más interesantes, más desarrolladas a lo largo de la actividad de formación y las que generan mayor interés desde el punto de vista de la realidad en la cual se desenvuelve el participante.
LOS TRABAJOS PRACTICOS GUIADOS.
g) Problemas presentados y sugerencias para mejorarlos en el futuro
Aspectos relacionados con la postulación al programa de formación o promoción
a) Apoyo de la Entidad Patrocinante (cuando corresponda) ____ bueno ____ regular ____ malo Justificar:
b) Información recibida por parte de FIA para realizar la postulación __X__ amplia y detallada ____ aceptable ____ deficiente Justificar:
c) Sistema de postulación al Programa de Formación o Promoción (según corresponda) ____ adecuado __X__ aceptable ____ deficiente Justificar:

d) Apoyo de FIA en la realización de los trámites de viaje (pasajes, seguros, otros) (sólo cuando corresponda)

__X__ bueno ____ regular ____ malo

Justificar:

e) Recomendaciones (señalar aquellas recomendaciones que puedan aportar a mejorar los aspectos administrativos antes indicados)

ANEXOS

1. RENDICIÓN DE GASTOS
2. BIBLIOGRAFÍA RECOPILADA
3. LISTAS ASISTENCIA ACTIVIDADES DE DIFUSIÓN

1. RENDICIÓN DE GASTOS

RENDICIÓN GASTOS PROYECTO FIA-FP-V-2004-1-A-034

ITEM	COSTO TOTAL	APORTE POSTULANTE	APORTE FIA	RENDIDO	DIFERENCIA
Pasaje Aéreos internacionales	$ 913.880	$ 0	$ 913.880	$ 913.880	$ 0
Pasajes Aéreos nacionale	$ 74.072	$ 0	$ 74.072	$ 74.072	$ 0
Tasas embarque	$ 76.756	$ 0	$ 76.756	$ 76.756	$ 0
Seguro de viaje	$ 78.740	$ 0	$ 78.740	$ 78.740	$ 0
Pasaje terrestre intern.	$ 124.000	$ 0	$ 124.000	$ 87.578	$ 36.422
Pasaje terrestre nacional	$ 32.000	$ 32.000	$ 0	$ 28.300	$ 3.700
Alojamiento	$ 595.200		$ 595.200	$ 351.000	$ 244.200
Viatico alimentación y mov.	$ 297.600	$ 297.600	$ 0	$ 297.600	$ 0
Matricula	$ 0		$ 0	$ 0	$ 0
Materales de trabajo	$ 310.000	$ 155.000	$ 155.000	$ 448.500	-$ 138.500
Material de difusion	$ 90.000	$ 90.000	$ 0	$ 79.895	$ 10.105
Gastos emision garantía	$ 48.000	$ 48.000	$ 0	$ 10.500	$ 37.500
SALDO					$ 193.427

TREN

= 50 shekel = € 12,5

TAXI "GORDON"
Rehovot Mall

מוניות "גורדון"
קניון רחובות

מזרחי דוד רח' יעקב 24
רחובות טל. 08-9456363, 052-2509921

עוסק מורשה 005095971

מקור
טל. 08-9451818

חשבונית מס/קבלה 3942

תאריך 2.03.05.

מונית מס' _____

שם הנוסע Miss Anna

עי"ח

אני מאשר בזה שנסעתי מ Rehovot

ל Ben-Gurion

רשימה _____

ש"ח
120

סה"כ כולל מע"מ

חתימת הנוסע _____

חתימת הנהג מזרחי דוד

120 + 30

TUR-BUS INTER JAC TUR

PASAJE TERRESTRE: Valido para la fecha y hora señalada. La devolución o cambio se aceptará hasta 4 horas antes del viaje (art. 67 D.S. 212/92 M.T.). Si Canceló en efectivo, se devolverá el 100 % del valor, si canceló con tarjeta (de credito, de debito u otra) se devolverá el 85 % del valor, si canceló con cheque se devolverá el 100 % del valor, 7 días después de la fecha de vencimiento del cheque, previa anulación del boleto antes del viaje, en oficinas con venta en línea. Si el valor de su equipaje excede de 5 UTM es su obligación declararlo previamente en la oficina de origen (art. 70 D.S. 212/92 M.T.). La Empresa no se hace responsable por perdidas que puedan ocurrir al interior del bus.

BOLETO VALIDO EN TUR-BUS

ORIGEN	DESTINO
OSORNO / CHILLAN	

Servicio a Bordo: DESAYUNO

Subida : Term de Buses Osorno / Bajada :

Cama

El horario de llegada es estimado. Los Boletos no confirmados tendrán una vigencia de 3 meses desde la fecha de venta.

(Bol. Pagado (0) Desc. ...)

Boleto Nro.A 41098136

Clave: 8...7

CODIGO DE CONFIRMACION:

CUENTA PREMIO TURCLUB

SALDO	:	
(HASTA DIA ANTERIOR A ESTA COMPRA)		
FECHA VIAJE	:	24/01/2005
DIA VIAJE	:	Lunes
HORA VIAJE	:	23:59
ASIENTO	:	15
VALOR $		14.400
VENDEDOR		kadg-osor a
F. VENTA		050121/15...

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TUR-BUS INTER JAC TUR

PASAJE TERRESTRE: Valido para la fecha y hora señalada. La devolución o cambio se aceptará hasta 4 horas antes del viaje (art. 67 D.S. 212/92 M.T.). Si Canceló en efectivo, se devolverá el 100 % del valor, si canceló con tarjeta (de credito, de debito u otra) se devolverá el 85 % del valor, si canceló con cheque se devolverá el 100 % del valor, 7 días después de la fecha de vencimiento del cheque, previa anulación del boleto antes del viaje, en oficinas con venta en línea...

BOLETO VALIDO EN TUR-BUS

ORIGEN	DESTINO
CHILLAN / OSORNO	

Subida : Term. Const/Brasil / Bajada :

El horario de llegada es estimado. Los Boletos no confirmados tendrán una vigencia de 3 meses desde la fecha de venta.

CODIGO DE CONFIRMACION:

CUENTA PREMIO TURCLUB

SALDO	:	
(HASTA DIA ANTERIOR A ESTA COMPRA)		
FECHA VIAJE	:	25/01/2005
DIA VIAJE	:	Martes
HORA VIAJE	:	11:55 AM
ASIENTO	:	40
VALOR $		5.300
VENDEDOR		cgcx-chll a
F. VENTA		050125/1148

Clasico

Boleto Nro.A 179365624

Clave: 617847

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$ 19.700.-


PASAJERO
Servicio de Minibuses Aeropuerto
Arturo Merino Benítez
0221354
TUR
Valor

LAN

GEORGE/ANJA

Vuelo/Flight Clase/Class Fecha/Date

LA 256 S 04FEB

OPERADOR LANEXPRESS

Desde/From Hacia/To Asiento/Seat

ZOS SCL 12B

Hora Embarque/
Boarding Time

17:45

Frequent Flyer

N° Pasaje/Ticket N° 0451663918391

Monto Pagado/Amount Paid CLP118129

Emitido/Issued At SANTIAGO CL

Fecha/Date 06JAN05

VARIG Brasil

NOME DO PASSAGEIRO
NAME OF PASSENGER

ECONOMY
GEORGE/ANJA

FQTV ##-NO.FQTV
EMPRESA CARRIER VÔO FLIGHT CLASSE CLASS DATA DATE

RG 8921 Y 05FEB

DE FROM PARA TO

SCL GRU NÃO FUMA NO SMOKING 0106 FUMO SMOKING

11 1515 16A NO

STAR ALLIANCE

VARIG Brasil

NOME DO PASSAGEIRO
NAME OF PASSENGER

ECONOMY
GEORGE/ANJA

FQTV ##-NO.FQTV
EMPRESA CARRIER VÔO FLIGHT CLASSE CLASS DATA DATE

RG 8756 Y 05FEB

DE FROM PARA TO

GRU LHR NÃO FUMA NO SMOKING 0146 FUMO SMOKING

2215 21A NO

STAR ALLIANCE

EL AL אל על

BOARDING PASS כרטיס עליה למטוס

NAME OF PASSENGER שם הנוסע

GEORGE A

FROM

TO

TEL AVIV

FLIGHT טיסה CLASS מחלקה DATE תאריך TIME שעה

LY0316 Y 06FEB

GATE 56 BOARDING TIME 1300 SEAT 49H

NO

שער PCS WT כוסף כרטיס עליה למטוס AGNT

LHR

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BOARDING PASS כרטיס עליה למטוס

NAME OF PASSENGER שם הנוסע

GEORGE/ANJA

FROM

2005

TO

FRANKFURT

FLIGHT NO. CLASS DATE TIME

LY0357 Y 02MAR

GATE BOARDING TIME SEAT

C06 0855 39A

PCS WT AGNT

1 25 E1 TLV

VARIG Brasil

NOME DO PASSAGEIRO
NAME OF PASSENGER

ECONOMY
GEORGE/ANJA

FQTV BB-NO.FQTV

EMPRESA CARRIER VÔO FLIGHT CLASSE CLASS DATA DATE

RG 8741 Y 05MAR

DE FROM PARA TO

FRA GRU

PORTÃO PORTA DE EMBARQUE GATE HORA DO EMBARQUE BOARDING TIME NÃO FUMANTE NO SMOKING FUMANTE SMOKING

0106

B28 2100 23F NO

STAR ALLIANCE

VARIG Brasil

NOME DO PASSAGEIRO
NAME OF PASSENGER

ECONOMY
GEORGE/ANJA

FQTV BB-NO.FQTV

EMPRESA CARRIER VÔO FLIGHT CLASSE CLASS DATA DATE

RG 8920 Y 06MAR

DE FROM PARA TO

GRU SCL

PORTÃO PORTA DE EMBARQUE GATE HORA DO EMBARQUE BOARDING TIME NÃO FUMANTE NO SMOKING FUMANTE SMOKING

0123

0900 19L NO

STAR ALLIANCE

LAN

EORGE/ANJA

Vuelo/Flight Clase/Class Fecha/Date

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00811130	קרם מזין	1	136.5	36	87.00
00811305	מלחי אמב	2	35.79	36	45.60

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*2505274633	ורסטיל EASY LEAD	6.60
*6381333658	טוש הדגשה בוס אד	3.40
96809116929	עטיפה 2 כיסים A4	3.20
	15.62%תיקון מחיר	0.50 -
	מחיר נטו 2.70	
*0006303813	מחברת תאקט חשבון	8.90
	44.94% הנחת פריט	4.00 -
	מחיר נטו 4.90	
	ל ת ש ל ו ם	17.60
	מזומן	20.00
	ע ו ד ף	2.40

סה"כ פריטים 4

ען 01 קוד 611 ת1609 16:26 07/02/05
קופאי/ת : יסמין תדמור-גולן
אקדמון מעמש בשלוח אוניברסיטה
חוברות אוני נמכרות ללא מע"מ

4,4

בס"ד

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טל 08-9343294 פקס 08-9343315
עוסק מורשה : 067860981
סניף : 1 מכון וייצמן

חשבונית מס/קבלה : 152428 מקור 20/02/05

עבור : 0 לקוחות שונים

תאור	כמות	מחיר	לתשלום
פיתות עבודת יד	1	6	6.00
החמוסיה עם טחינה	1	7.3	7.30
יופלה תות שדה	4	3.08	12.32
כללי	1	14.97	14.97
ג'אמפ לימון	1	5.5	5.50
קוטג תנובה 9%	1	4.87	4.87
סנו מקסימה 1.5 ק"ג	1	12.9	12.90

כמות פריטים :10 סה"כ : 63.86 ש"ח

סה"כ לתשלום כולל מע"מ : 63.90 ש"ח

שולם : 63.90 ש"ח
סה"כ לפני מע"מ 17% : 54.62 ש"ח
סה"כ מע"מ 17% : 9.29 ש"ח

עיגול סכומים : 0.04 ש"ח

פירוט תקבולים :

צורת התשלום	מספר	הסכום
מזומן		0.00
אשראי		63.9

13:36 קופאי :1 גיתית
קופה :1 ראשית
תם ספק :155394

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עוסק מורשה 0513046706

קוד	תאור	לתשלום
*2505163180	עט G-2 ג'ל לחצן	8.10

לתשלום 8.10

קוד	תאור	לתשלום
*0002219795	קפוצ'ון פליז NOS	43.10
	הנחת פריט 30.62%	13.20 -
	מחיר נטו 29.90	

לתשלום 38.00

ויזה 38.00

עודף 0.00

סה"כ פריטים 2

עמ 01 קופה 611 ח4669 09:04 23/02/05
קופאי/ת : יסמין תדמור-גולן
אקדמון משמש בשרות אוניברסיטה
חוברות אוני' נמכרות ללא מע"מ

אקדמון בע"מ
חנות רחובות
טלפון 08-9489463
עוסק מורשה 0513046706

קוד	תאור	לתשלום
39600002404	מפת טיול ישראל (	35.10

לתשלום 35.10

מזומן 50.00

עודף 14.90

סה"כ פריטים 1

עמ 01 קופה 611 ח2269 16:31 10/02/05
קופאי/ת : יסמין תדמור-גולן
אקדמון משמש בשרות אוניברסיטה
חוברות אוני' נמכרות ללא מע"מ

אסוציה ו' ש.עסקים בע"מ
ביוטי לירדי
הרצל 153 רחובות
טל 08-9478555
ח.פ. 513109907

01/03/05 10:04 00520013
מס' נורית 4297062

כמות	תאור/מחיר
	47.70

לתשלום:
47.70
מספר פריטים 1

שולם במזומן 50.00 ש"ח
עודף 2.30

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יוחננוף רכבת

כניסה צפונית
רחובות
טלפון ט 06-9475987
עוסק מורשה 0511344106

קוד פריט	תאור	לתשלום
2.10	X 5 יח'	
[illegible]	מיני תות	4.20
[illegible]	שמנת	1.85
244661	גלידה 80 גרם	8.90
[illegible]	פריכי [illegible]	3.10

לתשלום	18.05
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עודף	0.00

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	[illegible] גדול	1
10541000[illegible]		15.00

לתשלום	29.00
129.07 X מזומן	28.99
עיגול אגורות	0.01
עודף	0.00

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בירו מנטר
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האוניברסיטה העברית בירושלים
The Hebrew University of Jerusalem

February 10, 2005

המכון למדעי הצמח וגנטיקה בחקלאות
ע"ש רוברט ה. סמית
The Robert H. Smith Institute for Plant Sciences & Genetics in Agriculture

Mrs. Anja George
Avenida Rene Soriano 2363
Osorno
Chile.

Re: Plant Tissue Culture Training- Tuition Receipt

The sum of US $500.00 (five hundred) was paid by Mrs. Anja George for study - training in plant tissue culture from 06/02/05 to 02/03/05 at The Hebrew University of Jerusalem, in Prof. Ziv laboratory at the RH Smith Institute of Plant Science and Genetics in Agriculture, Rehovot Israel.

Professor Meira Ziv
Plant tissue culture laboratory.

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המזון ואיכות הסביבה
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Direccion: RUTA 215 KM 8
Giro: VIVERO
RUT:
Comuna: OSORNO
Guias N°

Por lo siguiente:

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FIRMA
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Dirección: Ruta 215 K. 8. — Ciudad: Osorno

Giro: Viveros — Teléfono: 230434

Guías Nº : — O/C Nº — Condiciones de Venta: Contado

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Son : Veinte mil pesos.

NETO $	16.807.-
19 % IVA $	3.193.-
TOTAL $	20.000.-

Nombre: Anja George
RUT.: 14.504.659-9 Firma:

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2. BIBLIOGRAFÍA RECOLPILADA

20. *In vitro* acclimatization

Meira Ziv

Department of Agricultural Botany and the Warburg Center for Biotechnology in Agriculture, the Hebrew University of Jerusalem, P.O. Box 12, Rehovot 76100, Israel

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1. Introduction
2. Plant structure and function *in vitro*
3. Effects of the culture microenvironment on plant development
   3.1. Mineral nutrient levels and composition
   3.2. Relative humidity (RH) in the culture headspace and medium water potential
   3.3. Gaseous components in the culture vessel
   3.4. Light and temperature
   3.5. Carbohydrates and growth regulators
4. Photoheterotrophic and photoautotrophic plant growth *in vitro*
5. Advanced technologies for acclimatization *in vitro*
   5.1. Controlled environment chambers
   5.2. Liquid medium as adjuvant or continual flow systems
   5.3. Acclimatization and rhizogenesis
   5.4. Induction of storage organs
   5.5. Growth retardants as acclimatization bioregulators
   5.6. Plant performance *ex vitro*
6. Conclusion

Key words: Automation, growth retardants, hardening, hyperhydricity, micropropagation, mixotrophic, nodular clusters, photosynthetic photon flux density, plugs, scale-up, transplanting

Species: cauliflower, chestnut, *Chrysanthemum*, *Delphinium*, *Dianthus caryophyllus* (carnation), *Geranium*, *Gladiolus*, *Juglans* (walnut), *Lilium*, *Lycopersicon* (tomato), *Malus* (apple), nerine, peach, *Philodendron*, *Picea abies* (spruce), *Pinus radiata* (pine), *Prunus* (plum; sour cherry), *Ptilotus*, *Rosa multiflora* (rose), *Rubus* (raspberry), saffron crocus, *Solanum tuberosum* (potato), *Spathiphyllum*.

1. Introduction

Plant propagation *in vitro* is currently an advanced biotechnological method to produce identical pathogen-free plants for agriculture and forestry. The method

J. Aitken-Christie, T. Kozai & M. Lila Smith (eds.), Automation and Environmental Control in Plant Tissue Culture, 493–516.

is still costly, and handicapped because intensive hand manipulation of the cultures is required, plant establishment is slow, and survival *ex vitro* is often low. Efficient commercial micropropagation depends on high proliferation rates during the multiplication stage, successful preparation of the plantlets during the acclimatization (hardening) stage and a high rate of plant survival, which must be coupled, to some degree, with automation.

The structure and function of micropropagated plants, as affected by various culture conditions unique to the *in vitro* environment, determines their ability to make the transition to the *ex vitro* environment. Plants grown in small culture containers *in vitro* are exposed to high levels of inorganic and organic nutrients, high relative humidity, elevated carbohydrate and growth regulator levels, low irradiance, limiting osmotic and water potentials in the medium, and limited CO_2 and O_2 gas exchange. These factors contribute to high proliferation rates, but also often induce physiological, anatomical and morphological abnormalities which interfere with the acclimatization and transplanting stages, and cause low survival rates *ex vitro* (Gaspar *et al.* 1987; Kevers *et al.* 1987; Ziv 1991a,d). The primary anomaly of plant structure and function that occurs *in vitro* has been recently defined appropriately as "hyperhydricity" (or hyperhydration) by Debergh *et al.* (1992). (Formerly, this phenomenon was called "vitrification" see Paques and Boxus (1987).) Hyperhydricity, which is characterized by various degrees of morphological and physiological disorders including a glassy, waterlogged tissue appearance and distorted growth, has mainly been reported in the shoot system, and more specifically in leaves (Ziv 1991a). Hyperhydricity influences photosynthesis, transpiration and CO_2 and O_2 gas exchange; dominant processes which could be detrimental to plant quality and survival (Kozai 1991d; Preece and Sutter 1991; Ziv 1991a). Other structural and functional abnormalities that have been cited for *in vitro* plantlets include sparse, underdeveloped leaf mesophyll, malfunctioning guard cells, and inferior vascular connections (roots and shoots).

The unfavorable consequences of the *in vitro* environment on plant development can be circumvented effectively by modifying the *in vitro* microenvironment to more closely parallel *ex vitro* conditions. By eliminating the abnormalities cited above via microenvironmental modification, a micropropagated plant's ability to survive after *ex vitro* transplant is improved. *In vitro* plantlets are known to have limited photosynthetic capacity, require sugars as an energy source and specific levels of nutrients and growth regulators. During hardening, a defined physical environment with controlled irradiance, gas (CO_2 and O_2) exchange and relative humidity are prerequisites for plant acclimatization (Kozai 1991d; Preece and Sutter 1991; Sutter *et al.* 1992). Current *in vitro* procedures for acclimatization are still unsatisfactory in providing quality micropropagated transplants for the greenhouse or the field. The alternative solution appears to be in providing culture conditions *in vitro*, prior to transplanting, which resemble photoautotrophic conditions *ex vitro*, and provide an optimal water balance for plant development (Kozai 1991b; Sutter *et al.* 1992). The procedures employed in conventional *ex vitro*

acclimatization – a gradual decrease in the relative humidity, removal of sugars, elevated irradiance and CO_2 – should be incorporated earlier in the production regime, during the preparation and hardening stages in culture. These modifications enhance the development of normal and adequate plant structure for efficient physiological functioning, improving shoot and root quality and increasing the survival rates of the plants *ex vitro*. Accelerated acclimatization *in vitro* can be achieved while the plants elongate and develop the shoot system, and the root system for some species. Modification of the *in vitro* production phases to more closely resemble *ex vitro* conditions will also contribute significantly to reduction in cost, resources, space and energy, and advance the achievement of economical micropropagation schemes.

This chapter describes the effects of culture conditions on plant structure and development, the significance of acclimatization *in vitro* for quality plant production prior to *ex vitro* transplanting, and various novel updated technologies which provide an improved *in vitro* environment that promotes superior plant development and performance *ex vitro*.

2. Plant structure and function *in vitro*

Clonal micropropagation of plants requires the establishment of culture conditions that promote extensive shoot proliferation. Culture conditions sustaining high shoot multiplication rates have a paramount effect on the anatomy, morphology and physiology of the developing shoots.

Leaves are the major organs affected during shoot development *in vitro*. Although stem structure is also affected, it has (unless very malformed), a smaller immediate impact on plant water stress and *ex vitro* survival. Roots, indispensable organs for a positive water balance, can be induced both *in vitro* and *ex vitro* by auxin (Preece and Sutter 1991), but the *ex vitro*-produced root system is far better adapted to survive acclimatization (Donnelly *et al.* 1985; Rogers and Smith 1992; Smith and McClelland 1991). Specific influences of the *in vitro* microenvironment on each of these organs are detailed below (Section 3).

Leaves of micropropagated plants which are confined in small, nearly airtight containers under low irradiance and high relative humidity were smaller, thinner, often translucent, and had poorly developed epidermal and mesophyll tissue. The cuticular layer in many species was reported to be structurally and chemically different from field grown plants (Cappelades *et al.* 1990; Grout and Aston 1975, 1977a; Sutter 1984, 1985; Sutter and Langhans 1979, 1982; Ziv *et al.* 1983). The stomatal apparatus in the epidermal tissue of micropropagated plants differed markedly from greenhouse and field grown plants. In geranium, rose, apple, and carnation, abnormally large stomata were observed (Blanke and Belcher 1989; Reuther 1988; Ziv *et al.* 1981, 1983, 1987). The guard cells were round instead of elliptic, raised above the epidermal surface and had thinner cell walls enclosing a large pore, which often remained

open (Donnelly and Vidaver 1984a,b; Lee *et al.* 1985; Preece and Sutter 1991; Wetzstein and Sommer 1982, 1983; Ziv *et al.* 1987). Stomatal density varied from one species to another, usually less densely scattered than in field grown plants (Preece and Sutter 1991; Smith and McCown 1983). Malfunctioning guard cells in several investigated plant species remained open even in darkness or under water stress conditions (Ziv 1991a). High CO_2, presence of ABA and Ca^{++}, as well as hypertonic solutions did not induce stomatal aperture closure in leaves or in isolated epidermal strips from cultured apple, plum, cauliflower and carnation plants (Brainerd and Fuchigami 1981, 1982; Brainerd *et al.* 1981; Fuchigami *et al.* 1981; Wardle *et al.* 1979; Ziv *et al.* 1987). Stomata from *in vitro Delphinium* leaves only partially reduced their aperture when exposed to closing signals (Santamaria *et al.* 1993). When CO_2-free air was applied to chrysanthemum plants partial closure was observed, however, neither darkness nor ABA induced stomatal closure (Wardle and Short 1983). Structural changes in the guard cells were accompanied by changes in the cell wall composition as revealed by epidermal histochemical studies. Lower levels of cellulose, pectins and cutin and elevated levels of callose were observed (Ariel 1987; Marin *et al.* 1988; Werker and Leshem 1987; Ziv and Ariel 1992, 1994). These were also accompanied by changes in the cellulose microfibril orientation revealed by the disoriented birefringence patterns (Fig. 1a,b) in guard cell walls (Ziv and Ariel 1992, 1994).

Changes in leaf epidermal cell structure inevitably affected their physiological function, manifested in the failure to control transpiration and in water imbalance. Cuticular and stomatal transpiration were higher in micropropagated than in field-grown plants. Leaves in transplanted apple plantlets lost water from the unclosed stomata at a significantly more rapid rate than the leaves of greenhouse grown plants. However, after about a week of acclimatization *ex vitro*, transpiration reached similar rates in the two plant groups (Brainerd and Fuchigami 1981). Potato plant transpiration *in vitro* was lower than in field grown plants (Kozai *et al.* 1992a), but when cultured under lower relative humidity the plantlets were more resistant to water stress without a decrease in dry weight (Tanaka *et al.* 1992). Shackel *et al.* (1990) calculated that the amount of water lost over a period of 24 hours, after removal of apple shoots from culture, was equivalent to 2–3 times the initial weight of the plants, although many of the stomata closed. Preece and Sutter (1991) state that "one cannot generalize that stomata of all micropropagated plants are unable to close". Differences in stomatal response which exist between excised and intact leaves (Sutter 1988) and different experimental procedures may explain some of the contradictory results.

In most species studied, the leaf mesophyll *in vitro* had a poorly developed palisade tissue, and was composed mainly of spongy parenchyma tissue with large intercellular air spaces (Brainerd and Fuchigami 1981; Grout and Aston 1978; Werker and Leshem 1987; Wetzstein and Sommer 1982). The mesophyll cells also had poorly developed chloroplasts, were low in chlorophyll and proteins and had disorganized grana (Cappelades *et al.* 1990; Lee *et al.* 1985; Wetzstein and Sommer 1983; Ziv *et al.* 1983, 1985; Ziv and Ariel 1992).

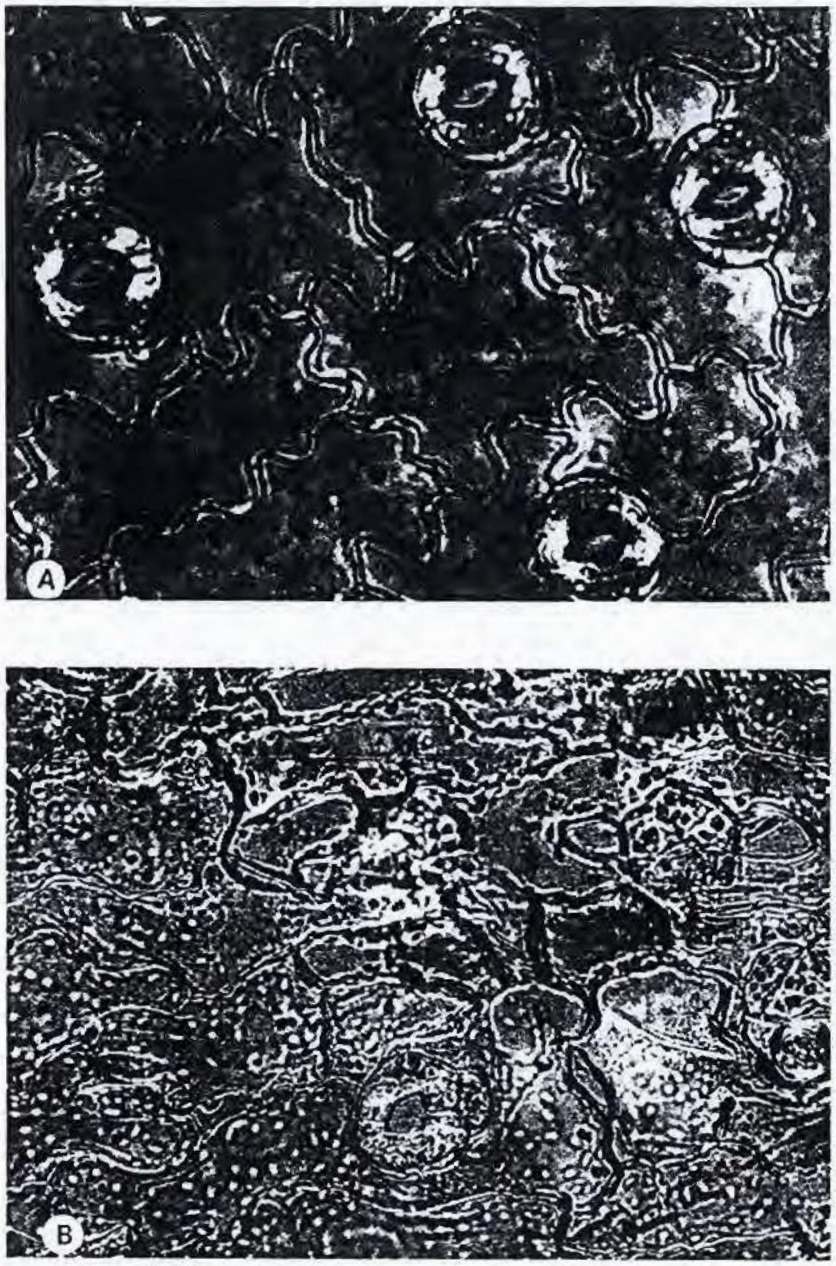

Fig. 1. Stomata from a normal carnation leaf showing typical guard cell wall microfibril orientation (a), and open malformed stomata from a hyperhydrous leaf lacking normal cell wall structure (b) (Ziv and Ariel 1992).

High relative humidity, low irradiance, the presence of high sucrose in the medium and inadequate CO_2 levels contribute to the heterotrophic growth of plants in culture. This is also due to poor chloroplast organization and inadequate metabolic activity of the photosynthetic enzymes (Desjardins *et al.* 1994, this volume). The uptake and fixation of CO_2 by *in vitro* plants is considerably lower in conventional agar cultures, in comparison to greenhouse or field grown plants (Donnelly *et al.* 1984; Grout and Aston 1977a; Reuther 1988; Tani *et al.* 1991; Watanabe *et al.* 1990). However, photosynthetic response curves estimation in several ornamental species showed that an increase in CO_2 and irradiance levels can enhance photosynthesis (Kozai 1991a).

Low ribulose biphosphate carboxylase (Rubpcase) activity contributed to the low rates of photosynthesis in cultured plant leaves (Grout and Aston 1977b; Grout and Donkin 1987; Grout and Millan 1985; Grout and Price 1987). Photosynthetic rates increased upon the removal of sucrose (Cappelades *et al.* 1990; Short *et al.* 1987) and the elevation of CO_2 (Desjardins *et al.* 1987, 1990; Kozai 1991b; Kozai and Iwanami 1988; Kozai *et al.* 1992a).

Plant stems developing *in vitro* are hypolignified, cell walls are thin, and there are large intercellular air spaces with a limited development of vascular tissue. Donnelly *et al.* (1985) observed that stems of micropropagated red raspberry plants had considerably less sclerenchyma and collenchyma supportive tissue than *ex vitro* plants. In chestnut, geranium and apple the stems lacked sclerenchyma tissue and the cortical and pith cells were hyperhydrated with large air spaces. Carnation stem vascular bundles lacked the normal organization (Werker and Leshem 1987), while in cauliflower, roots and stems, vascular connections were incomplete (Grout and Aston 1977a). In sour cherry, the xylem was functional and appeared to be continuous between the root and the shoot (Marin *et al.* 1988).

The usually agar-solidified root zone environment results in adventitious roots with poorly-developed vascular connections, little-to-no secondary thickening (for woody plants), a loose cortical cell arrangement, pigmented cells, and several other anomalous features which interfere with successful *ex vitro* acclimatization (Donnelly *et al.* 1985; Rogers and Smith 1992; Smith and McClelland 1991). As a result, only a percentage of the *in vitro*-initiated roots may survive *ex vitro* acclimatization, and depending on species, the original roots may be replaced with new *ex vitro* root initials (McClelland *et al.* 1990).

Provision of stress-free culture conditions, to overcome the complex nature of hyperhydration, must be emphasized in relation to the various metabolic pathways involved in plant morphogenesis and physiological function.

3. Effects of the culture microenvironment on plant development

The general features of the *in vitro* environment, its measurements and manipulation were reviewed in several papers by Kozai (1991a,b,c,d,e), Kozai *et al.* (1992a) and Ziv (1991a). The features entail a low flow rate of material and

energy, small fluctuations in temperature, high relative humidity, low irradiance, diurnal changes in CO_2, the presence of sugars, growth factors and regulators. In most cases, these unique, unnatural conditions effect the abnormal growth observed *in vitro*. However, the microenvironment can be manipulated and optimized substantially to sustain normal plant development *in vitro* and reduce the need for extended periods of usually stressful acclimatization *ex vitro* (Preece and Sutter 1991).

3.1. *Mineral nutrient levels and composition*

The medium most commonly used in conventional micropropagation at full, one half strength or any other combination of minerals, was developed by Murashige and Skoog (MS) (1962) for tobacco callus. It is high in nitrogen, potassium, magnesium and calcium and was found suitable for heterotrophic cultures. Several reports have suggested that shoot growth was better organized on half strength MS mineral salts medium (Kozai *et al.* 1988b, 1991). High levels of nitrogen in the NH_4^+ form were reported to cause hyperhydration, and hypolignification in several species. Plant morphogenesis was shown to improve and malformation to disappear in plants cultured in reduced MS salts or in media with lower levels of nitrogen (Ziv 1991a; Ziv and Ariel 1992).

The effects of Ca^{++} and Mg^{++} on plant development *in vitro* are contradictory; in some species high levels improved growth while in others high levels induced abnormal structure and function of the plants (Gaspar *et al.* 1987; Kreutmeier *et al.* 1984; Ziv *et al.* 1987). Supplementation of PO_4^{3-} during the latter stage of the culture period promoted the growth of strawberry plantlets (Kozai 1991d).

It seems that reducing the levels of some mineral notrients, to levels similar to media used in hydroponics, especially in scaled-up liquid cultures, can contribute to an improved morphogenetic response which provides for more efficient acclimatization (Kozai *et al.* 1992a; Ziv 1991a).

3.2. *Relative humidity (RH) in the culture headspace and medium water potential*

Plant growth and development can be severely influenced by high RH and media water potential. Temperature gradients inside the culture vessels and in the growth chamber may cause an increase of up to 95% RH in liquid or semi-solid agar media (Debergh 1987; Ziv 1986, 1991a). Various methods have been suggested to obviate detrimental effects of RH. In conventional micropropagation various gelling agents are used to support plants; their levels together with sugar and mineral concentrations will determine the water potential of the medium, the gradient between the medium and the culture atmosphere and thus the RH. Increased levels of agar – 1.0%–1.5% – provided an environment with lowered RH and supported normal growth (Debergh *et al.* 1981; Von Arnold and Erikson 1984; Ziv *et al.* 1983). The addition of desiccants,

PEG, a lanolin layer over the agar, or saturated salt solutions in the immediate environment to reduce RH have been shown to improve plant growth, wax deposition in the cuticle and stomatal function (Sutter and Langhans 1979, 1982; Wardle *et al.* 1983; Whish *et al.* 1992; Ziv *et al.* 1983).

The type of vessel closures used in conventional micropropagation to control contamination and desiccation prevent proper exchange of water vapor and other gases (CO_2, O_2, C_2H_4). Loosely capped culture vessels were shown to prevent hyperhydration by providing a better gas exchange (Blazkova *et al.* 1989; Dillen and Buysens 1989; Hakkaart and Versluijs 1983; Kozai and Sekimoto 1988; Ziv 1991a). Recently, the use of gas permeable films as enclosures, as well as forced ventilation were reported to improve shoot growth (Kubota and Kozai 1992; Tanaka *et al.* 1992a,b; Tsuji *et al.* 1992). Fujiwara *et al.* (1993), Kozai *et al.* (1992a) and Ibaraki *et al.* (1992) have demonstrated the dependence of the culture's RH on the number of air changes, on the gradient between the inside and outside environment of the vessels, and on leaf area of the plant.

3.3. *Gaseous components in the culture vessel*

The type and tightness of culture vessel enclosures determines the concentration of CO_2, O_2, C_2H_4 and water vapor in the culture gaseous atmosphere. In most cultures the CO_2 concentration, which was close to the compensation point, decreased during the light and increased during the dark period (Desjardins *et al.* 1987; Fujiwara *et al.* 1987; Pospišilova *et al.* 1992; Solárova 1989; Woltering 1986). These fluctuations were shown to exist also in loosely capped vessels with gas permeable membranes (Kozai 1991b). Under photoheterotrophic conditions of low irradiance and sucrose supplied as the energy source, the low level of CO_2 was not a growth limiting factor (Kozai *et al.* 1988, 1990). The introduction of advanced methods for the scale-up and automation of micropropagation imposes a demand for autotrophic or mixotrophic culture condition for quality plants (Aitken-Christie *et al.* 1992). CO_2 enrichment was shown to increase photosynthesis under elevated irradiance in several species, which was further improved by removal of sucrose from the medium at a reduced RH (Kozai *et al.* 1991; Nakayama *et al.* 1991). Various measures taken to enrich plant cultures with CO_2 suggested the use of a CO_2 control system in which CO_2 enrichment, along with gas exchange, could provide an optimal acclimatization microenvironment (Kozai and Iwanami 1988; Reuther 1988).

The concentration of other gaseous components, O_2 and C_2H_4, also depends on the type of vessel closures. Elevated levels of C_2H_4 caused hyperhydration (Gaspar *et al.* 1987; Hakkaart and Versluijs 1983; Ziv 1991a). Ventilation to prevent C_2H_4 accumulation prevented conditions which might otherwise cause development of aerenchymatous hyperhydrated tissue (Jackson *et al.* 1991).

Enhanced photosynthesis through the inhibition of photorespiration occurring under elevated O_2 levels, promoted normal shoot growth. Photosynthesis increased in both short- and long-term exposures of plants to 1–10% O_2 but not to 21% O_2 (Shimada *et al.* 1988). The levels of O_2 can also affect the

root system if anaerobic or low O_2 conditions prevail (Jackson *et al.* 1991). The control of the gaseous environment *in vitro* through gas permeable membranes and forced ventilation are most suitable for large-scale micropropagation.

3.4. *Light and temperature*

Photosynthesis and photomorphogenesis are radiation dependent: photosynthesis requirements are between 400–700 nm at high irradiance (PAR) while photomorphogenesis in the blue, red and far red regions' requirements are very low. Fluorescent lamps, commonly used in most tissue culture chambers, emit suboptimal irradiance, which is low in far-red wavelengths. Higher levels of radiation require costly cooling facilities which are often inefficient. Under low irradiation the plant may have an etiolated appearance. This condition is often due to the vertical radiation under which a radiation gradient forms. Several recent technologies aim at overcoming these problems (see Section 5). In most growth chambers the temperature fluctuates between the light and the dark periods around 22–26 °C providing a reasonable thermoperiod, however, the information on the effect of the temperature *in vitro* is inconclusive.

3.5. *Carbohydrates and growth regulators*

Mass propagation of plantlets depends on the presence of carbohydrates as an energy source for explant establishment and shoot proliferation stages. During the hardening stage, photoautotrophic and mixotrophic conditions can support plant growth (see Section 4). The most commonly used sugar is sucrose, although sometimes glucose and other sugars are used. Sucrose levels decrease during the culture period, usually after 2–3 weeks. Depending on the growth rate of the species it can reach very low levels, from an initial 3% to 0.2–0.5%.

Growth regulators, mainly auxins and/or cytokinins, are used for the establishment, proliferation and growth stages. During the acclimatization stage, auxins are used in conventional micropropagation to induce rooting *in vitro* prior to transplanting. In recent years, the trend is to avoid rooting in culture, to induce formation of root primordia only and to promote extensive rooting *ex vitro* (Preece and Sutter 1991).

Growth retardants from the triazol group, inhibitors of GA biosynthesis, have been implicated in acclimatization, promoting normal leaf structure and stomatal function (Roberts *et al.* 1992; Smith *et al.* 1990b; Ziv 1992; Ziv and Ariel 1991).

4. Photoheterotrophic and photoautotrophic plant growth *in vitro*

Micropropagated plants depend on the supply of carbohydrates as an energy source during multiplication and, in several species, during acclimatization. The developing plantlets are usually heterotrophic, have limited photosynthesis and

require gradual transition during acclimatization and after transplanting. Plant micropropagation under photoautotrophic indoor conditions, to provide quality plants prior to transplanting and reduce plant losses, has been proposed in recent reviews by Kozai (1991a,b) Kozai *et al.* (1992a) and Pospišilova *et al.* (1992) as a valuable strategy. Alternatively, the approach proposed by Debergh (1991) and De Riek *et al.* (1991) suggests that "photoautotrophy is not a must to acclimatize micropropagated plants, since heterotrophic plantlets can be acclimatized provided the right treatments are given" (Debergh 1991). The extent to which the "right treatments" are practiced, at what stage, and in which species, are probably where the main differences between the two approaches exist. Several studies to improve photosynthetic performance and growth of plants *in vitro* via control over the quality and quantity of irradiance, enrichment of CO_2 and increase in gas exchange were reported (Kozai *et al.* 1992a; Laforge *et al.* 1990; Williams *et al.* 1992). Frequently, this was accompanied by the reduction or removal of sucrose and control of RH. Cauliflower and raspberry plantlets *in vitro* showed low CO_2 fixation that probably improves in the newly developing leaves only after transplanting *ex vitro* to a sugar-free environment (Donnelly *et al.* 1984; Grout and Aston 1978; Grout and Donkin 1987). Contrary to these results, Short *et al.* (1987) reported that cauliflower and chrysanthemum plants grown on sucrose-free or sucrose-containing medium showed a higher photosynthetic rate on a sucrose-containing medium. The above inconsistencies could have resulted from differences in the experimental conditions and the type of leaves assayed, i.e., whether *in vitro* or *ex vitro*. In carnation, cymbidium and potato, removal or reduction of sucrose (to 1%) coupled with an enriched CO_2 atmosphere and elevated irradiance, induced an increase in the plants' dry weight (Kozai and Iwanami 1988; Kozai *et al.* 1988a,b). Photoautotrophic growth in sugar-low or sugar-free medium was also reported for *Rosa*, *Spathiphyllum*, *Pelargonium* (Reuther 1988) and *Vitis* (Reuther *et al.* 1992). Potato plants under elevated irradiance and in a CO_2-enriched environment were reported by Cournac *et al.* (1991) to behave photoautotrophically. The response, however, was cultivar dependent and they concluded that optimization must be adapted to the various cultivars and plants in culture. In freesia (Doi *et al.* 1992) and *Pinus radiata* (Aitken-Christie *et al.* 1992; Davies *et al.* 1992) growth was enhanced under elevated CO_2 and irradiance. On the other hand, Solárova (1989) presented evidence that under elevated CO_2 various measured growth parameters were higher in plantlets supplied with 1 or 2% sucrose. CO_2 enrichment to 350 ppm and elevated irradiance around 200 μmoles $\bullet$ $m^{-2} \bullet s^{-1}$ were an amended environment which provided photoautotrophic conditions for plant improvement (Kozai 1991a,c). Working with rose plants, Cappelades *et al.* (1990, 1991) found that the presence of 5% sucrose in the hardening stage medium improved leaf function. Carbon assimilated from the sucrose in the medium was stored in the leaves which later, during acclimatization, acted as storage organs. Furthermore, De Riek *et al.* (1991) showed that low net photosynthetic rates can be achieved during the multiplication stage under normal (non-CO_2 enriched, 3% sucrose, 35 μmoles $\bullet$ $m^{-2}s^{-1}$ radiation)

conditions, and it was coupled with a substantial increase in dry weight from CO_2 fixation. Obviously, the CO_2 released during respiration was sufficient to support photosynthesis.

These, as well as Kozai *et al.* (1988a, 1991) results indicate that *in vitro* plantlets are capable of photosynthetic activity providing CO_2 is supplied. However, the source of CO_2 varies: while in one approach CO_2 enrichment in the headspace should be from CO_2 gas inflow (Desjardins *et al.* 1990; Kozai *et al.* 1991), the other approach suggests the use of elevated sugars or repeated light/dark cycles in a 24 hr period to support high respiration rates and thus release CO_2 into the headspace (Cappelades *et al.* 1990; De Riek *et al.* 1991). Measurements of CO_2 in the headspace of several species cultured during the light period showed a sharp decrease from dark accumulated CO_2 within a few hours after the onset of the photoperiod (Kozai *et al.* 1992) as was also shown in tobacco *in vitro* (Solárova 1989).

A thorough study, including cost benefit calculation, should be conducted to determine which approach serves better the purpose of acclimatization *in vitro*, and whether the presence of sucrose inhibits or reduces photosynthetic activity in all species and types of tissues. It is difficult to assume that a small bud, having leaf primordia and limited leaf area during the multiplication stage, can develop without a carbon source. There is also the economic aspect of cost requirements to establish photoautotrophic conditions under elevated irradiance, for which cooling facilities are required.

5. Advanced technologies for acclimatization *in vitro*

The need for efficient and low production costs of quality micropropagated plants concomitant with robotization and automation to cut down labor intensive procedures, have motivated the advancement of novel biotechnologies for both the proliferation and acclimatization stages of micropropagation. Several innovative approaches were presented in a recently held symposium on "Transplant production systems" (Kurata and Kozai 1992). These systems included scaled-up cultures in bioreactors, controlled environment chambers, continuous flow of the medium using floating rafts, cultures with the double-layer liquid phase, bottom cooling in growth chambers, plug systems and the pre-initiation of root primordia, gas exchange films under forced ventilation, use of growth retardants, production of nodular clusters as well as *in vitro* corm, tuber and bulb formation in storage organ forming plants. Some of the methods will be discussed in relation to the acclimatization of plants *in vitro*.

5.1. *Controlled environment chambers*

Control of the microenvironment in the culture vessel can improve acclimatization procedures, enhance plant growth and increase plant survival *ex vitro*. A culture vessel incorporating a permeable membrane which allows gas

exchange and reduced RH was described by Roberts *et al.* (1992). The membranes permitted CO_2 exchange to support mixotrophic or autotrophic plant growth under high irradiance in the greenhouse. Such a system, using plugs, affords adjustments in plant acclimatization and eliminates the need for gradual adaptation *ex vitro*. A simpler system was described by Tanaka *et al.* (1992a,b) using a disposable film culture vessel which permitted improved gas permeability and high irradiance transmittance. Plants supported on rockwool under elevated CO_2 and sugar-free medium were photoautotrophic and significantly larger then the controls. An automatic *in vitro* liquid culture system monitoring RH, temperature, irradiance and CO_2 was used for carnation plants supported by sorbarod plugs. Ventilation through special filters in the containers provided better quality plants although it decreased the rate of multiplication (Majada *et al.* 1992). Such a monitoring system can provide information to help determine if growth, structure and function of persisting or newly formed leaves is improved. A microcomputer controlled acclimatization chamber has been developed by Fujiwara *et al.* (1988) and Hayashi *et al.* (1988). The unit provides controlled CO_2 levels, irradiance, RH, air temperature and air flow to plants in the culture vessels. Increased growth was observed after 4 weeks in strawberry plants cultured in this acclimatization chamber. Under a CO_2 enriched atmosphere the increase in dry weight was faster than the increase in leaf area when compared with conventionally propagated plants (Fujiwara *et al.* 1987, 1988). It is not clear whether the changes were monitored in newly-formed leaves, persisting older leaves, or in both. A further growth response was observed when irradiance was also elevated, thus enhancing autotrophy. In order to reduce the effects of irradiance heat, a novel lighting system was developed, using diffusive transparent plastic optical fibers, placed at the sides of the culture vessels and giving an even light distribution to the plant with minimal heat energy (Hayashi *et al.* 1992; Kozai *et al.* 1992b).

5.2. *Liquid medium as adjuvant or continual flow systems*

Plant acclimatization in liquid media, usually on some kind of a support system (filter paper, membranes, plugs) can provide a suitable microenvironment for the growth of root and shoot systems, eliminate the need for agar removal and decrease handling costs. Liquid medium can be supplemented as a second phase on the agar layer or introduced and removed automatically. Shoot elongation, rooting and overall enhanced growth were achieved efficiently by a double-layer technique introducing a liquid nutrient layer on top of the agar in herbaceous and woody plants (Aitken-Christie and Jones 1987; Maene and Debergh 1985). The system was further improved by using a transportable injector to introduce the medium through a septum in the lead, to cultures in the growth chamber, without the need to remove them to a laminar hood (Vanderschaeghe and Debergh 1987). A liquid overlay to facilitate hardening and rooting can improve acclimatization. Plants supported on glass beads, periodically flooded and drained in large culture vessels, responded with improved growth and

multiplication (Tisserat and Vandercook 1985). This was also found when liquid feeding was done by an automated misting system (Weathers and Giles 1988). Enhanced growth in a continual flow liquid nutrient bioreactor was achieved in herbaceous species cultured on a membrane. However, in some species, hyperhydration was observed, probably due to the direct contact with liquid medium in the membrane raft support (Hale *et al.* 1992).

There is no doubt that liquefied medium combined with a supportive system and a controlled microenvironment can produce normal, good quality, easy to handle plants. A microporous buoyant membrane raft developed to support plant growth in liquid medium in a membrane-vented culture box, seems to provide such a microenvironment (Tanny *et al.* 1993). These techniques should be further investigated.

5.3. *Acclimatization and rhizogenesis*

Micropropagated plants can be acclimatized *in vitro* simultaneous with rooting in an auxin-supplemented agar-solidified medium. Most shoots can be induced to initiate roots in culture, however the immediate contribution of fully developed roots to plant survival and low production costs are inconclusive and depends on the technique used and the species (Preece and Sutter 1991). Lack of functional vascular tissue with poor connection between the shoot and the root systems often restricts water uptake (Grout and Aston 1977a). In general, roots developed in agar are thin, and may thicken in high auxin media or in the presence of growth retardants (Roberts *et al.* 1992). In woody species, roots developed in agar are thick, have larger hypertrophied cortical cells and lack secondary vascular system (McClelland *et al.* 1990; Rogers and Smith 1992). *In vitro* rhizogenesis in herbaceous and woody plants showed a favorable shoot response for herbaceous species, while woody species, although they produced more roots per shoot, produced underdeveloped roots, with limited secondary vascular cambium activity, which was resumed only after *ex vitro* transplanting (Smith *et al.* 1991, 1992). Smith and McClelland (1991) showed that the choice of rhizogenesis method, *in vitro* or *ex vitro*, determines the long term structure and function of the root system.

Enhanced rooting and shoot growth were achieved in roses by a double phase of liquid-agar system (Smith *et al.* 1992) similar to the one reported by Maene and Debergh (1985) for several other species. Activated charcoal (Lilien-Kipnis and Kochba 1987) and CO_2 enrichment (Desjardins *et al.* 1987, 1990; Doi *et al.* 1992) also enhanced rooting. An auxin pre-initiation treatment for rooting apples, peaches and walnut microcuttings (Welander 1983; Zimmermann and Fordham 1985) induced root primordia. The pre-initiated root primordia developed into normal roots after transplanting and improved acclimatization. An efficient method using liquid media and plug systems enhanced rooting in chrysanthemum, gladiolus and cucumber (Roberts and Smith 1990; Ziv 1990, 1992b). In *Ptilotus*, reduced humidity and the presence of roots prior to transplanting increased plant survival (Whish *et al.* 1992).

5.4. *Induction of storage organs*

In several species belonging to the geophytes (storage organ producing plants) the need for acclimatization can be obviated by *in vitro* formation of tubers, corms or bulbs. Storage organs produced in culture survived transplanting better than plantlets; germinated in many cases without a cold treatment to break dormancy and developed normal shoots and roots (Ziv 1979, 1990; Ziv and Lilien-Kipnis 1990). Potato tubers were induced *in vitro* in an automated bioreactor system (McCown and Joyce 1991). Under specified photoperiod, high sucrose and ancymidol, microtubers were produced from randomly-cut shoots which were classified by machine vision prior to transplanting in a tuber induction medium (Alchanatis *et al.* 1994). Tubers can either be stored or planted directly and used as a source of pathogen-free propagation material.

Propagation of lilium bulbs initially developed in agar or liquid auxin containing medium were scaled-up in bioreactor cultures. These bulblets can be either transplanted or stored providing an efficient micropropagation system (Takayama *et al.* 1990). Large-scale bioreactors used for lilium bulb production yielded up to 800 bulblets per liter. These developed leaves upon transplanting *ex*

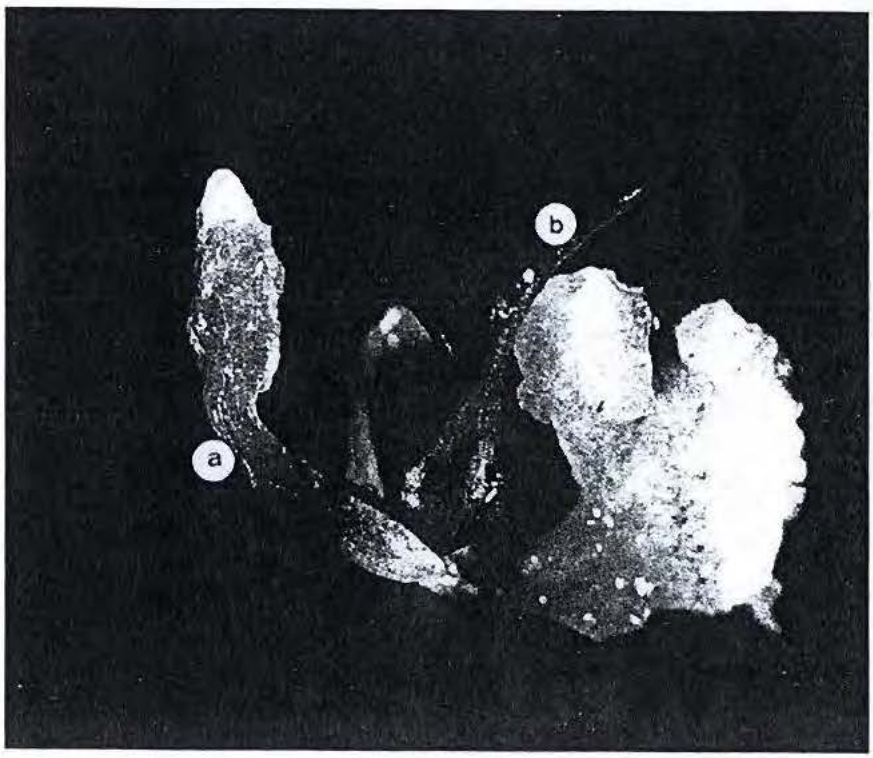

Fig. 3. A bulblet developing directly from a Nerine somatic embryo on 1/2 MS medium with 20 μM IBA (a) cotyledon (b) developing leaves (Ziv *et al.* 1994).

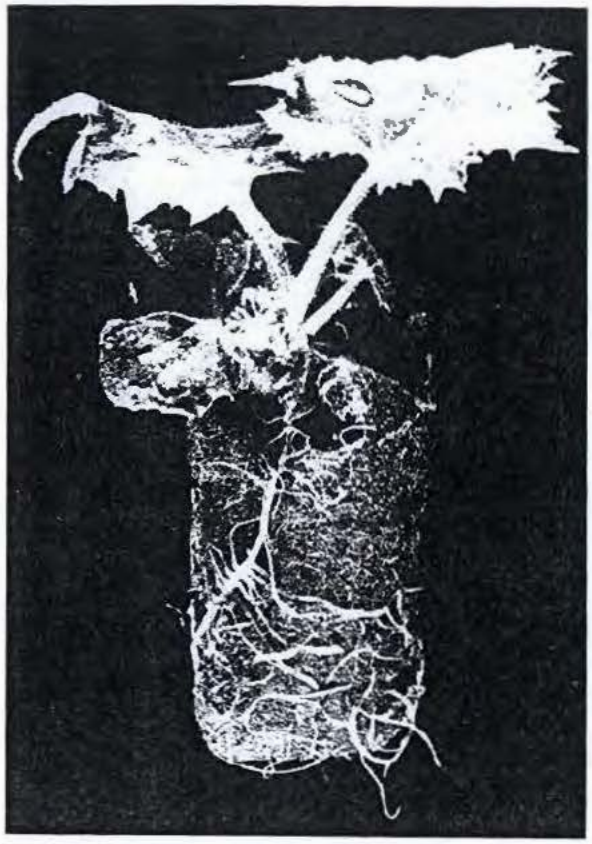

Fig. 2. A hardened cucumber plant with an extensive root system which developed from a somatic embryo and transplanted to a plug on 1/2 MS mineral medium with 0.4 μM ABA (Ziv and Gadasi 1986; Ziv 1992b).

Using a plug system for producing cucumber plantlets *in vitro* (Fig. 2) prevented root disturbance during transplanting as practiced in agar cultures, and improved root and plant survival *ex vitro* (Ziv 1992b). In most reports sucrose levels are usually reduced during rooting (Preece and Sutter 1991), however, in apple and pear microcuttings elevated sucrose during the multiplication stage improved rooting and survival *ex vitro* (Kunneman and Albers 1992).

If advanced micropropagation techniques are mandatory to obtain high quality micropropagated plants, an efficacious rhizogenesis system will contribute to plant survival. The liquid medium-plug system seems to be the most feasible system for efficient transition from *in vitro* to *ex vitro* acclimatization.

vitro without a need for cold treatment (Takahashi *et al.* 1992). Bulblets developed in Nerine from somatic embryos (Fig. 3) which were initiated in liquid medium on nodular clusters in the presence of paclobutrazol (Lilien-Kipnis *et al.* 1992). Corm development was reported in gladiolus on a high sucrose, auxin medium (Lilien-Kipnis and Kochba 1987; Ziv 1990) as well as in the presence of growth retardants (Steinitz and Lilien-Kipnis 1989; Ziv 1989, 1992a). Ziv (1990) found that *in vitro*-produced gladiolus corms germinated upon transplanting to the greenhouse without a need for cold treatment. Corms were produced in the saffron crocus (Plessner *et al.* 1990) on "Ethaphon" and kinetin containing medium and in freesia on a high auxin medium at 15 °C (Doi *et al.* 1992).

In geophytes, storage organ formation *in vitro* can be a potential strategy to overcome transplanting stress, increase plant survival and reduce plant losses *ex vitro*.

5.5. *Growth retardants as acclimatization bioregulators*

Growth retardants, inhibitors of gibberellin biosynthesis, are used extensively in agriculture and ornamental horticulture to control plant growth and structure, but until recently only to a limited extent in micropropagation. Growth retardants were reported to reduce shoot elongation and leaf area, to increase chloroplast content and improve stress resistance (Ziv 1991c, 1992a). Paclobutrazol and ancymidol controlled shoot growth *in vitro*, enhanced bud proliferation, and induced corm production in gladiolus (Steinitz and Lilien-Kipnis 1989; Ziv 1989, 1990). Paclobutrazol was found to increase desiccation resistance of micropropagated chrysanthemum, rose and grapevine (Roberts *et al.* 1992; Smith *et al.* 1990a,b, 1992). The effect of paclobutrazol was further increased in plants cultured in cellulose plugs with sucrose-free liquid medium under low RH. Desiccation resistance was associated with the increase of wax deposition, the improvement of stomatal response and the development of thickened roots (Novello *et al.* 1992; Roberts *et al.* 1992). In philodendron, paclobutrazol or ancymidol given during the proliferation stage in liquid medium enhanced plant survival *ex vitro* (Ziv and Ariel 1991). Growth retardants given at a suitable developmental stage and optimal level during acclimatization under photoautotrophic conditions may become promising bioregulators for *in vitro* plant quality.

5.6. *Plant performance* ex vitro

Reduced deposits of epicuticular waxes, the inability of the stomata of many micropropagated plants to close shortly after removal from culture, and deficient root systems are the major causes for water loss, desiccation and poor survival (Fabbri *et al.* 1986; Sutter *et al.* 1992). The photosynthetic capacity of transplanted plants, immediately after removal from culture, is probably not as crucial for survival as their ability to maintain a positive water balance. The

reports on the ability of leaves produced *in vitro* to revert to a fully functional state after transplanting are contradictory. Presumably, this may be the result of differences in the acclimatization procedures. Marin *et al.* (1988) and Sutter (1988) noticed differences in response to stress conditions between detached and intact leaves, a phenomenon that can explain the contradictory results. Shackel *et al.* (1990) found that irrespective of stomatal closure, plants lost water heavily over a period of 24 hours after transplanting. These and other results (Preece and Sutter 1991) indicate that cultured plant performance *ex vitro* varies greatly and at least during the first 2–3 days the ability of the plants to maintain a positive water balance is more pivotal than the photosynthetic performance. It should therefore be emphasized that *in vitro* acclimatization should provide a microenvironment to develop leaf and root structure that can withstand transpiration and support photosynthetic activity under stress conditions during the early phases of acclimatization *ex vitro*.

6. Conclusion

Plants developing *in vitro* are very often exposed to stress imposed by culture conditions such as limited space, low irradiance, high RH and improper gas exchange. This environment, chosen to enhance high multiplication, differs considerably from that provided to field or greenhouse-grown plants. Plants produced under such an *in vitro* microenvironment cannot survive transplanting and require gradual acclimatization to secure their survival.

In conventional *in vitro* acclimatization the need for a gradual decrease in RH, higher CO_2 and irradiance levels and depleted media is emphasized. Some of the methods employed to reduce humidity vary, from the use of desiccants and uncapping of the culture vessels for up to one week prior to transplanting (Ripley and Preece 1986; Wardle *et al.* 1979; Ziv 1986) to the method of bottom cooling to reduce the RH in the headspace of the container (Maene and Debergh 1987) or the use of appropriate ventilation (Kubota and Kozai 1992) and culture lids with permeable membranes (Tanaka *et al.* 1992a,b; Tanny *et al.* 1993). The introduction of advanced techniques for acclimatization *in vitro* include several strategies confronting irradiance, RH, CO_2 and other gases exchange, all aimed at producing photoautotrophic quality plants. Obviously, a thorough study of representative herbaceous and woody species, that compares at the same time the biological and economical aspects of autotrophic to mixotrophic growth is required.

Furthermore, as mentioned, plant photosynthetic performance is apparently second in importance to the water balance during the first 24–48 hours after transplanting. Therefore, the two physiological processes – transpiration and photosynthesis – should be investigated and monitored concomitantly. It is evident from the high survival rates reported for plants developing under photoautotrophic or photomixotrophic conditions (Cappelades *et al.* 1990; Desjardins *et al.* 1990; Dubé and Vidaver 1992; Grout and Price 1987; Kozai

1991c,e) that the environment which supported optimal photosynthetic capacity, is at the same time an environment that provides for developmental processes that allow for a positive water balance supporting normal plant structure. Under these conditions the headspace RH, a major factor in the development of the cuticular and stomatal structure, as well as the photosynthetic apparatus are controlled. It is appropriate in the present context to consider introducing photoautotrophic conditions during the multiplication stage. However, if large scale cultures are to be introduced as part of an automated micropropagation system, then the requirement for autotrophic conditions when using nodular clusters in liquid media is inconclusive (McCown and Joyce 1991; Ziv 1991b, 1992a).

All of the foregoing evidence gives further support to the innovative new technologies such as forced ventilation, diffusive films, continual or intermittent nutrient flow, CO_2 enrichment, growth retardants, and rhizogenesis in plug systems as contributing factors to a controlled microenvironment which will sustain economical production of quality micropropagated plants.

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MASS PROPAGATION OF FERNS THROUGH TISSUE CULTURE

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Abstract

The present investigation deals with rapid mass propagation of three different varieties of ferns namely: Pteris vittata, Nephrolepis exaltata and Cyathea gigentia, employing tissue culture technology. By adopting this procedure large number of superior and uniform quality of these ferns were produced throughout the year.

1. Introduction

Ferns are cultivated in public parks, nurseries and as indoor plants because of their evergreen ornamental foliage. Recently, their demand from nurseries, tissue culture laboratories as well as from private agencies far exceeds their supply. Besides, the conventional methods employed for the propagation of ferns which included layering or cutting of stolon or rhizome segments from parent plants during the rainy season. But the ferns produced by this method developed single growth centre from where few fronds emerged out. Murashige (1974) successfully propagated many varieties of ferns on mass scale, employing tissue culture technology. Caponetti (1978) emphasized the rapid propagation of saleable ferns was achieved by in vitro culture of excised leaf primordia. Besides, Boxus (1978) reported that many of the horticultural establishments have gradually adopted in vitro multiplication techniques.

The present investigation deals with the standardisation of tissue culture procedures for rapid mass propagation in Pteris vittata, Nephrolepis exaltata and Cyathea gigentia throughout the year.

2. Materials and methods

In Pteris vittata, healthy rhizome segments with apex, stolon segment in Nephrolepis exaltata and leaflet primordia and apical domes of Cyathea gigentia were excised from garden grown plants. These explants were surface sterilized with mercuric chloride (0.1 or 0.01 %) for few minutes then washed several times with sterile distilled water. Each of the explant was cut into desirable size and inoculated on the culture medium. The various culture media employed

were White's medium (1954), B5 Gamborg's e t a l. medium (1968) and Knudson's medium modified by Steeves e t al. (1955). Each of the medium was supplemented with sucrose (2 to 4%) and various growth regulators such as napthelenacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), Indolebutyric acid (IBA), Kinetin (K) and 6 benzylaminopurine (BAP) as per the requirement of the experiments. In White's medium for callus induction coconut milk (CM) was added. The pH of the medium was adjusted to 5.7. All the cultures were incubated in continous flourescent light (1000 Lux) at 25±2°C.

3. Results

In P t e r i s v i t t a t a, rhizome segments with anex, cultured only on White's medium containing sucrose (4%) and K (0.5 mg/l) showed excellent proliferation and produced 10 - 12 new shoots within four weeks. These shoots, on separation and transfer to fresh medium of the same composition, each one, once again produced a fresh crop of new shoots. These shoots when well developed were transferred to rooting medium containing sucrose (0.5%) and NAA (0.2 mg/l). Thus large number of plantlets were raised.

In second set of experiments, callus was raised from P t e r i s v i t t a t a rhizome segments by culturing them on White's medium containing sucrose (2%), 2,4-D (2.0 mg/l) and coconut milk (Figure 1). To investigate the morphogenetic potential of this callus, it was cultured on sucrose (4-5%) containing medium. Large number of shoots regenerated from the callus mass (Figure 2). These shoots were allowed to grow further and transferred to the rooting medium. By this procedure also, number of plants were produced. These regenerated ferns showed diploid chromosome number. But on the whole, as the number of subcultures of callus on 2,4-D containing medium increased, the morphogenetic potential of the callus decreased.

In N e p h r o l e p i s e x a l t a t a, stolon segments (1 to 2 cm) cultured on B5 medium containing sucrose (3%) and IBA (1.0 mg/l) produced greenish outgrowths along their margin within a week. Microscopic examination of these structures revealed that they were, the meristematic growth centres. At this developmental stage, these cultures were transferred to B5 medium containing K (0.5 mg/l) and BAP (0.5 mg/l). Within four weeks in response to the cytokinin treatment, the growth centres, further developed into numerous adventitious buds. These buds were dark green structures easily separable. Each bud on separation and grown on the fresh medium of the same composition, produced large number of buds. Each bud, on transfer to the basal medium produced a plantlet (Figure 3). By this technique about 10,000 N e p h r o l e p i s plants were resulted from a single stolon segment within four months. Further, experimental work on the regeneration of new offsprings from the original stolon

explants indicated that they should not be cultured indefinitely to raise these ferns. Every now and then fresh stock of plant should be used.

Experiments with C y a t h e a g i g e n t i a were conducted on excised young leaflet primordia and apical meristem. They were grown on modified Knudson's medium supplemented with sucrose (3%), K (2.0 mg/l) and NAA (0.1 or 0.2 mg/l). Each of the leaflet primordium grew faster and developed into complete frond. These well developed fronds were transferred to rooting medium. Moreover, the apical dome grown on Knudson's medium containing sucrose (4%) and K (2 mg/l) and NAA (0.2 mg/l) enlarged and buds were induced in places of leaf primordia. In this fern the apical meristem was surrounded by six young leaf primordia, and hence, seven propagules could be obtained from each apical dome. Each of this transformed bud was excised and cultured on the same medium when it developed into a plantlet (Figure 4).

All these i n v i t r o produced ferns were hardened, within the culture flasks which facilitated their transfer to soil. Further transfer of these plants was done using mist chamber.

4. Discussion

The present experimental work indicated that the mass propagation of these ferns was possible, independent of the season. These results indicated that the physiological age of the explant greatly influenced its morphogenetic capacity (Padhya and Mehta, 1982). The youngest, least differentiated leaf primordia exhibited highest ability for morphogenesis.

There are hardly any reports about induction of adventitious buds by cytokinin treatment in ferns hence this work in N e p h r o l e p i s serves impetus for conducting experimental work on other fern genera. Moreover, each fern species responded well to specific medium which showed that the nutritional requirements varies from plant to plant. In P t e r i s v i t t a t a, regeneration of plant was possible from the rhizome callus but production of plants without intervening callus phase is much desirable to maintain uniformity in the ferns produced.

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Fig. 1. P t e r i s v i t t a t a rhizome callus grown on White's medium containing (2%) sucrose, (2.0 mg/l) 2,4-D and (10%) Cm.

Fig. 2. *Pteris vittata*, shoots regenerated from the rhizome callus when grown on basal medium containing (4%) sucrose.

Fig. 3. Plantlets of *Nephrolepis exaltata* regenerated from adventitious buds.

Fig. 4. *Cyathea gigentia* plantlet during transfer to pot.

PROPAGATION OF THE FERN *ADIANTUM CAPILLUS-VENERIS* THROUGH TISSUE CULTURE OF THE CIRCINATE PART OF YOUNG LEAVES

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Abstract

The circinate part of young leaves,excised before the beginning of foliar expansion,was inoculated on Gamborg B_5 medium supplemented with auxins IBA(0.05 to 4.0mg.l^{-1}) or NAA(0.05 to 4.0mg.l^{-1}) or 2,4-D(0.5 to 2.0mg.l^{-1})and cytokinin BA(0.05 to 5.0mg.l^{-1}).Combinations of different concentrations of IBA anb BA as well as of 2,4-D and BA were tested.

The best morphogenic response was obtained with Gamborg B_5 medium supplemented with IBA 0.5mg.l^{-1} and BA 0.01mg.l^{-1},2%sucrose,at pH 5.5. After 8-10 weeks of culture,formed meristems were transferred to fresh medium without growth regulators(developing medium).Six weeks after,plantlets were transferred to the rooting medium(Gamborg B_5 plus NAA 0.05mg.l^{-1}).Well developing plantlets,suitable for potting,were obtained after, at least,1 month on this medium.A condition for plant growth is the soil composition and a relative high atmospheric humidity(about 100%).

1. Introduction

Ferns,namely *Pteris cretica* var. *albo-lineata*, *Nephrolepis* sp.and *Adiantum peruvianum* have been cultivated for their ornamental foliage.

The great part of experiments were carried out using rhizome apices. In what concerns the propagation of *A.peruvianum*,different types of explant have been used such as petioles,leaves and apical meristems of rhizomes (Morel,1956).In this case,only apical meristems could regenerate plants.

Our paper deals with the clonal propagation of *Adiantum capillus-veneris* using as explant,the circinate part of young leaves.

2. Material and Methods

The young leaves of *Adiantum capillus-veneris*, belonging to the fern collection of the Lisboa Botanical Garden,were washed in running water and surface desinfected in a 10% calcium hypochlorite solution for 5min. The circinate part of young leaves was cut and inoculated on solid (0.7% Difco Bacto agar)Gamborg B_5 medium (1968)supplemented with auxins B-indolbutyric acid(IBA)or naphtalenacetic acid(NAA) at the concentrations 0.05,0.1,0.5,1.0,2.0,4.0mg.l^{-1} or 2,4-dichlorophenoxiacetic acid (2,4-D) at the concentratitions 0.5,1.0 and 2.0mg.l^{-1} and benzyladenine (BA)at 0.05,0.1,0.5,1.0,2.0,5.0mg.l^{-1}.Other media were prepared using the following combinations of IBA and BA(a-IBA 0.05/BA 1.0;b-IBA 0.1/BA 1.0;c-IBA 0.5/BA 1.0;d-IBA 1.0/BA 1.0;e-IBA 0.5/BA 0.5;f-IBA 1.0/BA 0.5 ;g-IBA 0.5/BA 0.1;h-IBA 1.0/BA 0,1;i-IBA 0.5/BA 0.05;j-IBA 1.0/BA 0.05; k-IBA 0.5/BA 0.01 and l- IBA 1.0/BA 0.01mg.l $^{-1}$)or 2,4-D(1.0mg.l^{-1}) and BA(0.5,1.0,2.0mg.l^{-1}). Sucrose 2 and 3%)and glucose 2% were tested as carbon source; pH values between 4.5 and 6.0 were assayed.

A Gamborg B5 medium with sucrose 2% , with and without yeast extract

$0.5g.l^{-1}$ at pH 5.5 was used as a development medium. A rooting medium (Gamborg B_5 plus $0.05mg.l^{-1}$ NAA, with or without yeast extract $0.5g.l^{-1}$) was used. For comparison, the Murashige and Skoog (1962) medium (MS) supplemented with IBA $1.0mg.l^{-1}$ was used. Gamborg B_5 media including NO_3^-, NH_4^+, Ca^{2+}, Cl^- at the MS molarities were also tested.

The incubation was carried out under 16h light/8h dark photoperiod (1700 lux) at $24\pm2°$. A soil with humus, fertiliser, turf and sand (4:2:2:1) and a very high atmospheric humidity (about 100%) was used for potting.

3. Results and Discussion

The Gamborg B_5 medium has been widely used for plant propagation. In what concerns the propagation of *A.capillus-veneris*, the circinate part of young leaves (fig.1) inoculated on this medium supplemented or not with growth regulators (NAA, IBA and/or BA) could express morphogenic response. If 2,4-D is used alone or combined with BA, only callus could be obtained The Gamborg B_5 medium used without growth regulators promoted the formation of 15 to 20 meristems. However, they are very easily damaged when transferred to the rooting medium. The efficiency of this procedure was about 30%. Wetmore (1954) using apical meristems of *A.pedatum* cultivated on Knop solution, has reported that auxins can be omitted from the medium with no serious loss, but producing plants with less vigor.

When the medium contains IBA or NAA, at concentrations between 0.05 and $1.0mg.l^{-1}$, meristems developed after 10-12 weeks. Plantlets are more vigorous than those produced in the absence of growth regulators (fig.3). At this developmental stage, they can be transferred to the rooting medium, the efficiency of the procedure being about 40%. From the two auxins used, IBA has revealed better than NAA because it produces greater number of plantlets and roots are less vigorous. In order to prevent formation of roots (fig.4) that difficult separation of plantlets, combinations of different concentrations of IBA and BA, representing BA/IBA ratios between 20 and 0.01 (from medium a to medium l) were tested. The best morphogenic response was obtained with medium k. Six weeks after inoculation, the first meristems appeared (fig.2) and two weeks later, so many meristems was formed that it was difficult to count them (fig.5). The foliar primordia appeared after two more weeks, inspite of the formation of new meristems (fig.6). All the other media (a to j) produced meristems but they failed to form leaves. Medium l only promoted the formation of a small number of meristems.

The results obtained with these media shows that the number of meristems increase with decrease of the ratio BA/IBA. This morphogenic response and that obtained with the absence of growth regulators, seems to indicate that the leaves of this fern possess an endogenous supply of auxins and cytokinins, probably being higher the amount of cytokinins. Wetmore et al. (1949) has suggested the synthesis of auxins by *A.pedatum* leaves.

The meristems formed 8-10 weeks after inoculation, are transferred to the development medium in order to stop the formation of new meristems and to induce plantlet differentiation (fig.7). Six weeks later each plantlet was transferred to the rooting medium were vigorous plantlets, suitable for potting, developed after 1 month (fig.8). The supplementation of this medium with yeast extract is recommended because it promotes the formation of greater number and better developed leaves. In the rooting medium, NAA must be kept at $0.05mg.l^{-1}$ which is the appropriate concentration for quick root development. Similar effect of yeast extract and NAA was reported for development of *A.pedatum* plantlets obtai-

ned from rhizhome aplices (Wetmore,1954).

Plantlets obtained were successfully transferred to the prepared soil in the presence of high atmospheric humidity for the first weeks (fig.9).

In what concerns the nitrogen source, our experiments revealed that it must not be used at molarities higher than those of Gamborg B_5 medium. Higher molarities of NH_4^+ or of NO_3^- promoted the formation of abnormal leaves. According to Wetmore(1954), the development of *A.pedatum* is faster in the presence of nitrogen sources other than nitrates. These results suggest the high molarities of nitrogen sources used in the MS medium can presumably be the reason for the effectiveness of this medium for *A. capillus-veneris* propagation.

4. Conclusions

From our results we can conclude that: 1) the circinate part of *A. capillus-veneris* young leaves present high capacity to differentiate on the Gamborg B_5 medium supplemented with NAA,IBA and or BA;2) the procedure more suitable for efficient propagation is:(a) inoculation of the circinate part of young leaves on medium k, (b) transfer of meristems to developing medium, (c) transfer of plantlets to the rooting medium, (d) potting of plantlets at the described conditions; 3) all the plants propagated according to this procedure exhibited uniformity in their quality; 4) the efficiency of the above procedure (65%) to produce great number of plantlets (about 130) and the short period of propgagation (five months until potting), allied to the non damage of the mother plant (as it arrives if rhizome apices are used as primary explant) may contribute to commercial feasibility of this procedure by fern growers.

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Legendes of figures

Fig. 1 - Circinate part of young leaf with appropriate development to be inoculated, x 90

Fig. 2 - Meristem formation 6 weeks after inoculation on Gamborg B_5 plus 0.5 mg. l^{-1} IBA and 0.01 mg. l^{-1} BA, x 108

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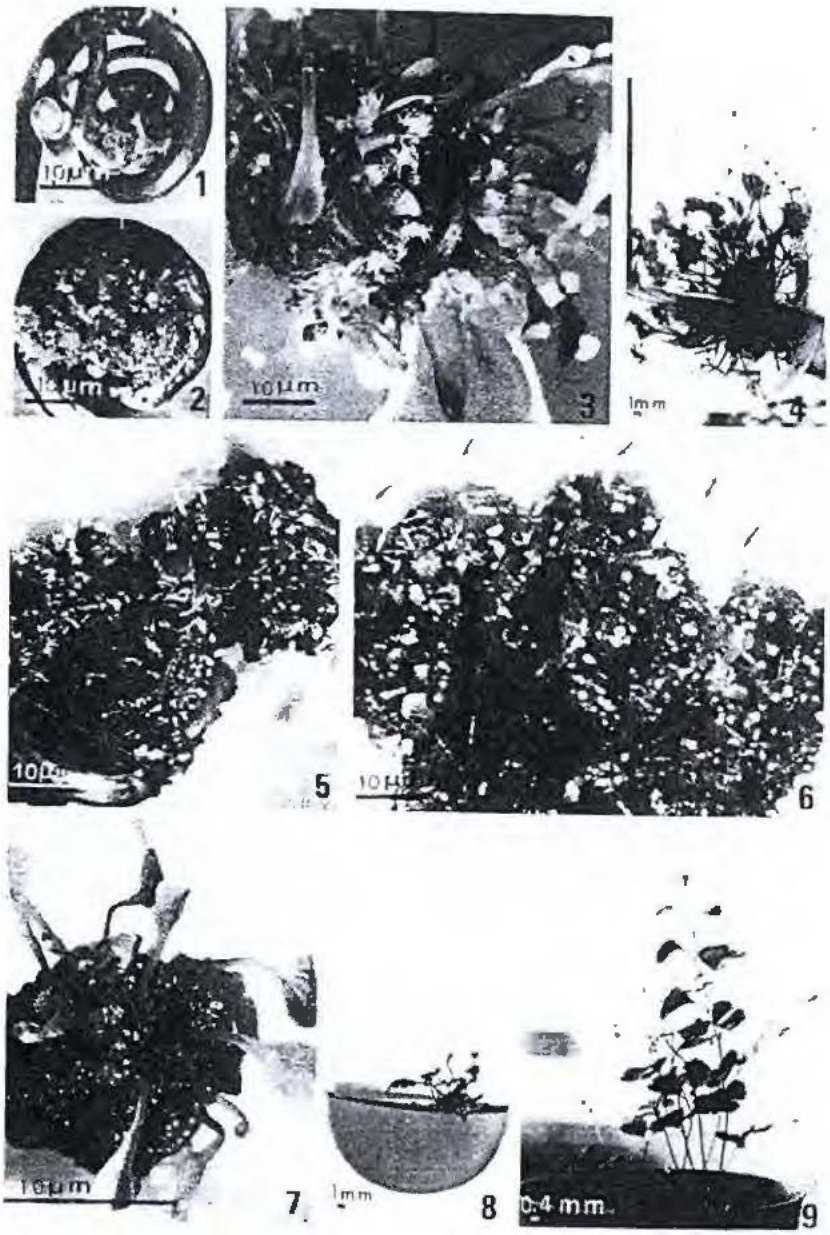
Fig. 4 - Plantlets obtained on the medium of fig. 3, 16 weeks after inoculation. Note the great number of roots(arrows), x 1.3



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CLONAL MASS PROPAGATION OF THE FERN CYRTOMIUM FALCATUM

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Abstract

Collected spores of *Cyrtomium falcatum* were sterilized according to Knauss method and germinated in an inorganic liquid medium as formulated by Dyer in 1969. The germination of the spores were observed within 7 to 9 days, and after 3 to 6 weeks both gametophytic and sporophytic stages were produced. Several gametophyte bearing sporophytic tissue were selected and divided in 4 to 6 sections. Each of these sections were cultured on Murashige and Skoog medium as revised by Leinsmaier and Skoog in 1965, and the effect of different substances on the regeneration of the complete plant was then analized. We tested sodium phosphate monobasic (255 mg/l), L-cystein (30 mg/l) and different concentrations of auxins: naphtaleneacetic acid (NAA), indole 3-butyric acid (IBA); and cytokinins: kinetin (K), 6-bencylaminopurine (BAP). Sucrose (20g/l) was added to all media. The induction of the formation of fast growing tissue with high regenerative characteristic was achieved either with K (2 mg/l) and ANA (0.1 mg/l) or with BAP (3 mg/l) and ANA (0.1 mg/l), but a better proliferation was observed in the medium with K and ANA. Differentiation of shoots and complete plantlets were obtained in a medium with lower salt concentration (Gamborg medium) and substituting IBA for ANA. For better elongation of the leaves gibberelic acid, GA (0.1 mg/l) was added. Between 30 to 50 plantlets per month were obtained in each flask after the regenerative tissue was established. The final amount depends on the size of the containers.

1. Introduction

For many years ferns were propagated by traditional methods using spores, rhizome sections, runners and others. The application of tissue culture methods to propagate ferns was first reported by Wetmore and Morel (1949), who obtained roots, leaves and rhizomes by culturing apical meristem of *Adiantum pedatum*. Presently, many commercial laboratories are using tissue culture techniques to produce large quantities of ferns by clonal propagation.

Knauss (1976) described a partial tissue culture method to propagate *Crytomium falcatum* and other fern species. He got gametophytic tissue under aseptic conditions, which was sectioned and transfered to soil to produce new plants. In this investigation a complete tissue culture method has been developed to get rapid clonal multiplication of *Cyrtomium falcatum*.

2. Materials, Methods, Experiments

2.1 Plant material, media and culture methods

Spores were collected from sporophylls of *cyrtomium falcatum*. They were freeded of any other vegetal material and sterilized according to Knauss method (1976). After sterilization, the spores were cultured in inorganic liquid medium as formulated by Dyer (1979), or on solid medium based on Murashige and Skoog salt mixture (1962) with 30gr/l sucrose and 0.8% Difco Bacto-Agar.

To get sporophytes, once the gametophytic stage was developed, several gametophyte bearing sporophytic tissue were divided in 4 to 6 sections, each of them were cultured in 250 ml flask containig solid Murashige and Skoog medium as revised by Linsmaier and Skoog, (1965), with

30gr/l sucrose and without growth substances.

To establish a fast growing tissue (proliferating tissue), some of the young sporophytes were taken out of the culture vessel, cut in 4 sections and transfered to fresh nutrient media containig inorganic nutrients recommended by Murashige and Skoog (1962), minor organics such as 0.4 mg/l, and 100mg/l myo-inositol (RM medium), and supplemented with 20 mg/l sucrose, 255 mg/l phosphate monobasic, 30 mg/l cystein and different concentrations of growth substances: cytokinins (kinetin, K and 6-benzyl amino purine, BAP) and naphtaleneacetic acid, NAA. (Table 1).

Shoot morphogenesis from that proliferating tissue was achieved by transfering many small pieces of this tissue to the same basic medium (RM), or to half strengh RM medium, suplemented with BAP, K and NAA in different concentrations (table 2). Developing shoots were isolated and cultured on Gamborg et al (1968) salt solution (G medium), plus NAA, gibberelic acid (GA) and indole 3-butyric acid (IBA) for root initiation and shoot enlargement (table 3).

All media were adjusted to pH 5-8 prior to solidifying with 0.8 % Difco Bacto-Agar and autoclaving at 121 ^{0}C and 15 lbs inch^{-2} for 15 minutes.

Tissues were incubated in a growth chamber, in constant light (white fluorescent lamps, daylight type, giving 2600 lux) at 28 ^{0}C.

2.2 Growth estimation

The percentage of explants forming proliferating tissue was determined after one month of growth. The regeneration frequency (percentage of proliferating tissue sections developing shoots) as well as the number of shoots showing a rooting response were also determined one month after the subculture in each treatment was established.

Anatomical features were examined from 3 month old plants. Roots and leaves samples of both types of plants (one obtained by tissue culture method, and the other by germination spores in soil) were preserved in 70% alcohol to make free-hand sections. The sectioned material was stained with 0.05% toluidine blue. Micrographs were taken from the prepared slides.

2.3 Transfer of plantlets to soil

Rooted shoots were transfered to small plastic vessels containing sterilized prepared soil and placed in a propagator with low light intensity and relatively high humidity enviroment. After two weeks they are transfered to a shaded place at atmospheric conditions.

3. Results

3.1 Formation of sporophytes under sterile conditions

The germination of spores of *Cyrtomium falcatum* and the initiation of the sporophytic stage were achieved on the Dyer medium. No suger was needed to germinate the spores. To complete the development of the sporophytes, sections of gametophyte bearing sporophytic tissue were subculture on RM medium and after several weeks many sporophytes were produced.

3.2 Establishment of the proliferating tissue

Sporophyte sections developed on the edges of leaves, a very fast growing tissue with high morphogenic capacity. This tissue was produced when sections were cultured on RM media supplemented with 2 mg/l K and 0.1 mg/l to 0.2 mg/l ANA, or 3 mg/l BAP and 0.2 mg/l ANA. The highest percentage of explants forming proliferating tissue (70%) was observed when they were treated with 0.1 mg/l ANA and 2 mg/l K (Table 1). Shoot formation was also observed in this treatment, but at very low frequency. After one month it lost organization and became proliferating tissue.

Sections of sporophytees obtained from young sporophytes grown in soil, were also used as

explants to initiate proliferating tissue, but no growth was observed.

3.3 Shoot formation and multiplication

The proliferating tissue had a very high regenerative characteristic and was able to develope shoots even on a medium with 0,1 mg/l ANA, but the regeneration frequency was improved when ANA was eliminated from the medium (Table 3).

The composition of the medium where the proliferating tissue was initiated had effect on the regeneration frequency of the tissue. Proliferating tissue initiated on medium with 0.1 mg/l ANA and 2 mg/l K, produced 100% of shoot when they were subcultured to media containing 2, 1 or 0.5 mg/l K. Tissue initiated on medium with 0.2 mg/l ANA and 3 mg/l BAP produced 20% of shoots, only when they are treated with 1 mg/l K. The latter regeneration frequency was improved when the tissue were subculturde two times on the same medium.

The morphogenic capacity of the proliferating tissue was not decreased by the number of subculture provided the tissues were grown on the appropiate medium. In this way it was possible to get a large scale producción of *Cyrtomium falcatum* via shoot formation from proliferating tissue.

3.4 Rooting and enlargement of shoots

Shoots were formed as a cluster of rossettes without roots. For rooting and enlargement of shoots it was necessary to subculture these rossettes on a medium with lower salts concentration (Gamborg), supplemented with IBA or ANA, both with or without GA (Table 3). Rooting of the shoots was achieved in all the treatments. Shoot elargement and leaf expansion were improved on media with GA.

Plantlets were removed from agar, potted in sterile soil and placed under a high humidity condition. After two weeks, they were transfered to a shaded place under normal enviromental conditions. All plantlets grew properly in soil.

Histological analysis showed that the anatomical features of the leaf of both types of plant (grown in aseptic condition and grown in soil) are similar, but the number of layers of mesophyll cells increases in plants produced by tissue culture. The number of layer of cortex cells in roots of in vitro plants decreases.

4. Discussion

It was possible to produce large numbers of *Cyrtomium falcatum* plants from sections of in vitro grown sporophytes, that developed a rapid proliferating tissue with high morphogenic capacity. This tissue differentiated many shoots when cultured on RM medium with Cytokinins and without auxins. Sporophytes sections excised from young sporophytes grown in soil did not proliferate on culture conditions. It seems that the nutrients of the culture medium created endogenous condition that prepared tissues to respond to growth induction.

Shoots were produced as clusters of rosettes and did not form roots. To induce enlargment of shoots and root formation, rosseles were isolated and cultured on G medium containing 1mg/l IBA and 0.1mg/l GA. This medium has lower salts concentration than MS medium, and promoted the rapid initiation of root system. According to Murashige (1979) high salt levels suppress rooting. A lowering of salt concentration may be required when Murashige and Skoog medium is used. Shoots grown on media with GA produced higher plantlets with wider and larger leeves. Engelke it al (1973) showed that the size and form of shoots depends on GA/ cytokynins ratio, high ratios resulted in tall, spindly plants wiht narrow leaves while low ratios resulted in short shoots with rounded leaves. It is possible that the new *C. falcatum* plantlets have enough endogenous concentration of cytokinins to balance the amount of GA added exogenously.

The method described in this peper allows rapid clonal propagation of *C. falcatum* by using

tissue culture techniques. The advantage of this method is the obtainment of proliferating tissue with high morphogenic capacity which preserves its regeneration frequency independently of the number of subcultures. Once this tissue is established it can be transfered to a maintenance medium to lower growth rate or can be grown on media for multiplication and differentiation to obtain many plantlets that can be adapted very easily to soil conditions.

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Figure 1 - In vitro growing sporophytes

Table 1 - Induction of proliferating tissue formation.

Medium	Substances added mg/l				% of sections with proliferating tissue	% of sections with shoots
	NAA	BAP	K	$NaH_2PO_4H_2O$		
RM_1	0.1	–	2	–	70	33,3
RM_2	0.2	–	2	–	40	0
RM_3	0.2	3	–	255	50	0

Table 2- Shoot development from proliferating tissue sections.

Medium	Subtances added (mg/l)				% of sections with shoots	
					Initiated in medium	
	NAA	BAP	K	$NaH_2PO_4H_2O$	RM_1	RM_3
RM_4	–	–	2	–	100	0
RM_5	–	–	1	–	100	20
RM_6	–	–	0.5	–	100	0
RM_7	0.1	–	2	255	12,5	0
$^1/_2RM$	0 1	–	2	–	16	0
RM_t	0.1	–	2	–	50	–
RM_3	0.2	3	–	255	–	0

Table 3 - Rooting and enlargement of shoots.

Medium	Growth substances (mg/l)			Leaf expansion (cm)		Root formation
	NAA	GA	IBA	width average	length average	
G	–	–	1	0 91	0 91	plenty
G_1	–	0 1	1	1 29	1.23	plenty
G_2	1	–	–	0 93	0 91	plenty
G_3	1	0 1	–	1 22	1.23	plenty

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Control of Oxidative Browning and *in Vitro* Propagation of *Strelitzia reginae*

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Additional index words bird-of-paradise, tissue culture, oxidative browning

Abstract. **The use of tissue culture techniques for the propagation of *Strelitzia reginae* Banks has invariably failed due to oxidative browning of the explants. The brown exudate which diffuses into the medium is detrimental to further development of the *in vitro* explant, which eventually becomes necrotic and dies. *S. reginae* competence to respond to *in vitro* culture is thus dependent on minimizing and slowing down the browning of the explant. Terminal and axillary buds treated with antioxidant solution by total submergence for 24 hours, and cultured on an agar medium with charcoal, or on paper bridges in liquid medium with dithiothreitol, were found to be capable of growth and further shoot proliferation.**

S. reginae, an important cut flower of South African origin, is a perennial monocotyledon with a short, subterranean stem. The terminal bud produces 10 to 14 leaves before dichotomous branching occurs. The 2 shoot systems continue to develop independently, each capable of producing leaves and new axillary buds, forming a large clump with many shoots (2). New plants are propagated from seeds or vegetatively by the division of old plants. Both propagation methods are very slow. Seedling plants are variable, and flowering occurs only after 4 to 7 years. For division, plants at least 10 years old are used, each producing only a few units. This explains why *Strelitzia* is one of the very few important cut flower plants for which no uniform cultivars are available commercially. Development of a reliable propagation method can contribute to the selection of improved clones and the propagation of clonal material for this plant.

Plant propagation *in vitro* has been established successfully for a large number of monocotyledonous ornamental plants (3), including *Musa cavendishii* (7). In most plants propagated *in vitro*, success depends on proper explant selection, medium composition, and suitable procedure for transplanting and establishment under nonaseptic conditions (9, 10). The use of tissue culture techniques for the propagation of *S. reginae* has invariably failed due to oxidative browning of the wounded explant. The brown exudate that diffuses into the medium was found to be detrimental to further development of the explants, which eventually become necrotic and die. This paper describes *in vitro* methods for reducing oxidative browning and increasing the propagation potential of *S. reginae*.

Plants were grown in pots in a plastic screen shade (20%) structure under natural conditions. Prior to explanting *in vitro*, the older leaves and root system were removed and the shoots were rinsed under running water for 24 hr. The leaf blades and all but the basal 5 cm of the petioles were removed, and the remaining condensed stem was rinsed in 70% alcohol for 5 min. The condensed stem was then disinfected for 15 min in 9% $Ca(C1O)_2$, or in 0.3% $HgCl_2$, followed by one rinse in sterile distilled water. The plant material then was totally immersed in a solution containing antioxidants for 12, 24, 48, or 72 hr. The autoclaved solution consisted of Murashige and Skoog (MS) liquid medium (8), excluding the vitamins, sucrose, and growth substances, to which were added (per liter) 150 mg citric acid, 100 mg ascorbic acid, 5 mg each of filter-sterilized chloramphenicol and oxytetracyclin, and 25 mg benomyl. The pH was adjusted to 4.5 prior to autoclaving at 121°C and 1.1 kg/cm² for 15 min. Following pretreatment, the plant material was rinsed once in sterile distilled water and twice in 1% ascorbic acid. Explants were excised on a filter paper soaked in either 1% ascorbic

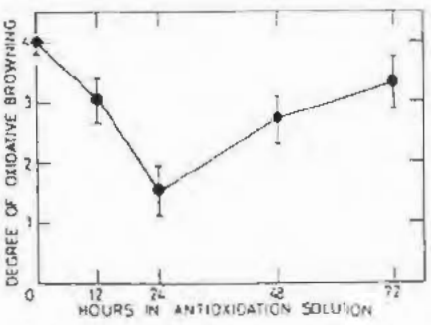

Fig. 1. The effect of immersion of *S. reginae* shoot tip explants in a solution with antioxidants. Rating scale: 1 = browning on excised wound tissue; 2 = partial browning on outer tissue of explant; 3 = browning of the entire explant tissue; 4 = browning and necrosis. Data points are means of 12 explants ± SE.

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acid or 0.04% dithiothreitol (DTT). Explants were isolated from the terminal bud, from the nodal tissue of the stem including 2 leaf bases 0.4- to 0.5-cm-long (similar to "twin scales" in bulbs), and from the inflorescence stalk. Both solid and liquid modified MS media were used with half-strength macroelements. In liquid cultures, the explants were supported on filter-paper bridges. The liquid medium contained 0.04% filter-sterilized DTT, while the agar-solidified medium was supplemented with 1% activated charcoal.

Different combinations of growth substances were tried, of which the following levels (in mg/liter) were found to be best for initial culture: indolebutyric acid (IBA) 2.5, naphthaleneacetic acid (NAA) 1.0, kinetin 5.0, and 2,4-dichlorophenoxyacetic acid (2,4-D) 0.5. Phloroglucinol (150 mg/liter) was added to the agar-solidified medium in order to enhance terminal and axillary bud growth. All cultures were incubated for 10 days in the dark and then were placed under 14-hr daily illumination (1.3×10^{-3} Wcm^{-2}) supplied by Grow/VHO Sylvania fluorescent tubes in a culture room maintained at 24 ± 1°C.

The main problem in the *in vitro* culture of *S. reginae* was to reduce oxidative browning and to activate shoot growth before necrosis extended from the accompanying tissue to the developing buds. Oxidative browning of wound tissue was less severe when 0.3% $HgCl_2$ was used instead of $Ca(ClO)_2$. Soaking the explants in pretreatment solution containing citric and ascorbic acid at pH 4.5 for 12 hr decreased browning only slightly, while 24 hr immersion reduced oxidative browning considerably (Fig. 1). Prolonged immersion for 48 or 72 hr was not effective, possibly because of submergence under anaerobic conditions, which may cause the production of toxic substances. Prolonged immersion often resulted in contamination of the explants, despite the presence of antibiotics and a fungicide in the solution. Maintaining the explants in darkness reduced the diffusion of a brown exudate into the culture medium. Similar findings were also observed in *Eucalyptus* (1). Although browning and some necrosis developed in the surrounding tissue, the buds continued to grow (Fig. 2A, 2B). Soaking in a low pH solution further reduced the oxidation of polyphenols released by the wound tissue, apparently by inhibiting the activity of phenolases (6). Explants not pretreated with antioxidants became brown during excision. It was necessary to excise the various explants on filter paper soaked with ascorbic acid or DTT; the latter was found to be more effective for reducing browning of wounded tissue.

Various amounts of callus were observed to develop on explants from the young inflorescence stalk and the stem nodal tissue (Table 1, Fig. 2A). Both liquid (A) and agar-solidified charcoal medium (B) stimulated callus production after 4 weeks in response to kinetin and the 3 auxins (Table 1), while medium C with phloroglucinol was not effective. The callus continued to grow rather slowly for several weeks without organogenesis, which also was not observed upon subculture to different media with various combinations of growth substances. Charcoal medium was found to support the best initial growth of both terminal and axillary buds (Table 1; Fig. 2C). In the axil of the leaf bases, 3 to 4 vegetative buds developed. As can be seen in Fig. 2C, the buds originated one next to the other; they are not the result of a single bud proliferating. Unlike *Cinchona* explants (4), *S. reginae* explants on a medium to which only phloroglucinol was added did not show enhanced bud growth and became necrotic in the absence of charcoal or DTT. Stimulation of axillary bud growth was observed when explants, which were cultured initially for 3 weeks on charcoal medium (B), were subcultured to a liquid medium (A) in which NAA and 2,4-D were excluded, kinetin was substituted by 1.0 mg/liter benzylamino purine, and IBA was increased to 5.0 mg/liter. As can be seen in Fig. 2B, the bud in the axil of the leaf bases developed despite the browning and necrosis of the surrounding tissue. The bud reached a height of 3 cm after 40 days. Since only one root at most developed in culture, the

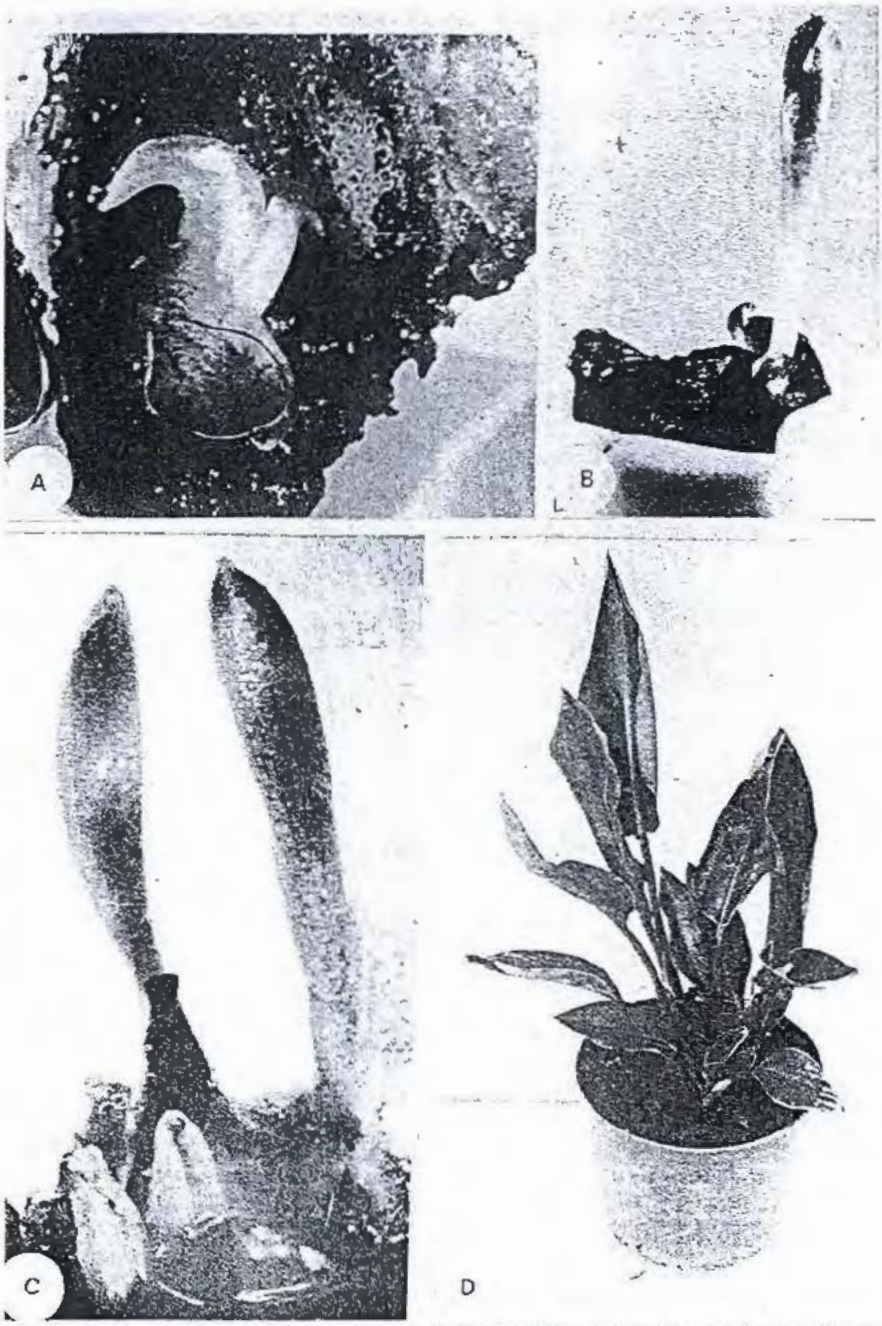

Fig. 2. *In vitro* development of *S. reginae*. A. Callus production and bud growth from nodal stem tissue on charcoal medium (B — see Table 1) after 45 days in culture (×3.6). B. Axillary bud growth after subculture from a charcoal medium (21 days) to a liquid medium. Note bud growth in spite of stem nodal tissue necrosis (× 3). C. Vegetative bud development on charcoal medium after 78 days (× 2.8). D. Cluster of 18-month-old *S. reginae* shoots which developed from an axillary bud initiated on charcoal medium and transplanted after 78 days to liquid medium with BA and IBA, and 6 months later to the greenhouse (×0.45).

Table 1. Medium effect on oxidative browning, callus production, and shoot development from different *S. reginae* explants.

Explant	Medium[a]	Degree of[b] browning	Callus[c]	No. of shoots ± SE
Terminal bud	A	2	++	2.1 ± 0.1
	B	1	++	4.2 ± 0.2
	C	3	−	1.0 ± 0.3
Stem nodal tissue with leaf bases	A	2	++	3.0 ± 0.3
	B	1	++	4.8 ± 0.5
	C	4	+	1.3 ± 0.3
Inflorescence stalk	A	2	+++	−
	B	2	+++	−
	C	4	+	−

[a]Medium A = liquid medium + DTT, explants supported on paper bridges; Medium B = agar medium + charcoal; Medium C = agar medium + phloroglucinol. All media contained (mg/liter) 5.0 kinetin; 2.5 IBA; 1.0 NAA; 0.5 2,4-D.
[b]Rating scale: 1 = browning on excised wound tissue; 2 = partial browning on outer explant tissue; 3 = browning of the entire explant tissue; 4 = browning and necrosis.
[c]Callus: − (none); + (low); ++ (medium); +++ (high).

shoots were treated as cuttings (5) and were soaked in autoclaved 100 ppm IBA for 4 hr, prior to transplanting to an autoclaved mixture of 1 peat : 1 vermiculite. Although shoot proliferation was limited to 5 new shoots at most, the *in vitro* development of vegetative buds in the axils of the leaves could increase the propagation potential of this species. Multiple bud development in the leaf axil could have resulted from the early isolation of the nodal tissue explant before flower differentiation, and in response to the 3 auxins in combination with kinetin. After transplanting and establishment under nonsterile conditions, the cluster of shoots elongated and developed (Fig. 2D).

Antioxidants used prior to *in vitro* culture of *S. reginae* and the addition of charcoal or DTT to the culture medium have effectively controlled oxidative browning and stimulated *S. reginae* shoot growth.

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האוניברסיטה העברית בירושלים

הפקולטה למדעי החקלאות, המזון ואיכות הסביבה
המכון למדעי הצמח וגנטיקה בחקלאות

קורס 71138 – תרביות רקמה של צמחים – עקרונות ושימושים (תשס"ד)

פרופ' אריה אלטמן

להלן רשימת ספרות רקע בסיסית בנושא שיטות עבודה בתרבית רקמה להשלמה עצמית, הכרת העבודה היא חיונית להבנת נושאי הקורסים.

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האוניברסיטה העברית בירושלים
הפקולטה למדעי החקלאות, המזון ואיכות הסביבה
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פרופ' אריה אלטמן

רשימת ספרות – מקורות לאיזודים ומאמרים לקריאה

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QUALITY OF MICROPROPAGATED PLANTS—VITRIFICATION[1]

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Summary

Vitrification-Hyperhydrous shoot development, effects the survival and quality of several micropropagated plants ex-vitro. The leaves which are the immediate organ to be affected, exhibit abnormal morphology and physiology. Leaf malfunction is apparently a stress response to very rich media and high relative humidity. The understanding of the underlying mechanism of vitrification and its control in vitro can contribute to a more efficient micropropagation. Vitrification was found to be associated with elevated ethylene production which was related to hypolignification and poor cell wall development. Liquid and low agar media induced callose formation along with reduced and disoriented cellulose biosynthesis, manifested also in non-functioning guard cells. Malfunctioning stomata, in addition to defective cuticle contributed to increased transpiration and desiccation of in vitro formed leaves. The activity of various enzymes, associated with cell wall synthesis, was low and total proteins in normal leaves was higher than in vitreous ones. Various measures were found to reduce vitrification; lowered matrix and water potential in the medium, reduction in RH, low NH_4^+, changes in Ca^{++} levels and the removal of ethylene. These measures improved leaf morphogenesis, survival and the quality of several micropropagated plant species.

Key words: acclimatization; agar; Ca^{++}; cell wall; epicuticular waxes; ethylene; hyperhydration; lignification; micropropagation; NH_4^+; peroxidases; relative humidity; stomata; vitreous leaves.

Introduction

Understanding the various factors involved in the control of plant growth in vitro can greatly improve the quality of micropropagated plants and prevent undesired shoot development known to impede plant survival ex vitro.

Structural disorders in micropropagated plants are manifested by anatomical, morphologic, and physiologic anomalies observed in shoots cultured under conditions required for rapid bud proliferation and growth. The disorders are termed vitrification, translucency, hyperhydration, succulency, and glassiness and refer mainly to leaf appearance. Anomalous anatomy is manifested to a lesser extent also in the stems and roots (10,16). Complex, multiple factors interrelated through various metabolic pathways in vitreous leaves affect the two major processes carried out by the leaves: photosynthesis and gas exchange. These disorders, resulting from the special requirements for shoot proliferation in vitro, cause shoot malformation and as a consequence impede micropropagated plant establishment ex vitro (53).

Shoot Anatomy and Morphology In Vitro

Although vitrification is usually described by the glassy water-soaked appearance of the leaves (Figs. 1 *a* and 2 *a*), various anatomical and physiologic changes were reported to precede or occur concomitantly with malformed shoot development (22,36,53). The hyperhydrous mesophyll in the leaves of several species consists of a poorly developed palisade tissue and a spongy parenchyma with extensive intercellular air spaces (15,18,25,29,37).

Changes in the epidermis of vitreous leaves were observed both in the cuticle and the guard cells. Reduced deposition of epicuticular waxes, both in quantity and quality, was reported in cauliflower, cabbage, crysanthemum, carnation, raspberry, and apple and was found to relate to the water status of the cultures (5,14,17,42–45,53). In carnation, decreasing the relative humidity in the culture vessel induced normal leaf development (Fig. 1 *b*) and an increase in epicuticular wax formation (Table 1) (13,53).

Abnormal leaves from various species cultured under high relative humidity in vitro, had large malformed stomata (1,3,51) that did not close in response to darkness, CO_2, abscisic acid (ABA), hypertonic solution, or a water stress (55). In carnation the abnormally large guard cells were rounded instead of eliptical (Fig. 1 *c,d*) and had large pores, thin cell walls, and fewer chloroplasts (59).

Histochemical studies of guard cells from vitreous carnation leaves revealed lower levels of cutin, pectins, and cellulose (1,26,51,57). Callose deposits in guard cells from vitreous leaves were reported in cherry and carnation, and the cell walls lacked the normal orientation of cellulose microfibrils typical of guard cells (1,34,55,57,59). In vitreous carnation leaves, Ziv et al. (59) have shown that the defect in the guard cells lay in the cell walls. The failure of the cell walls to contract in hypertonic solution was shown to correlate with the abnormal orientation of cellulose microfibrils

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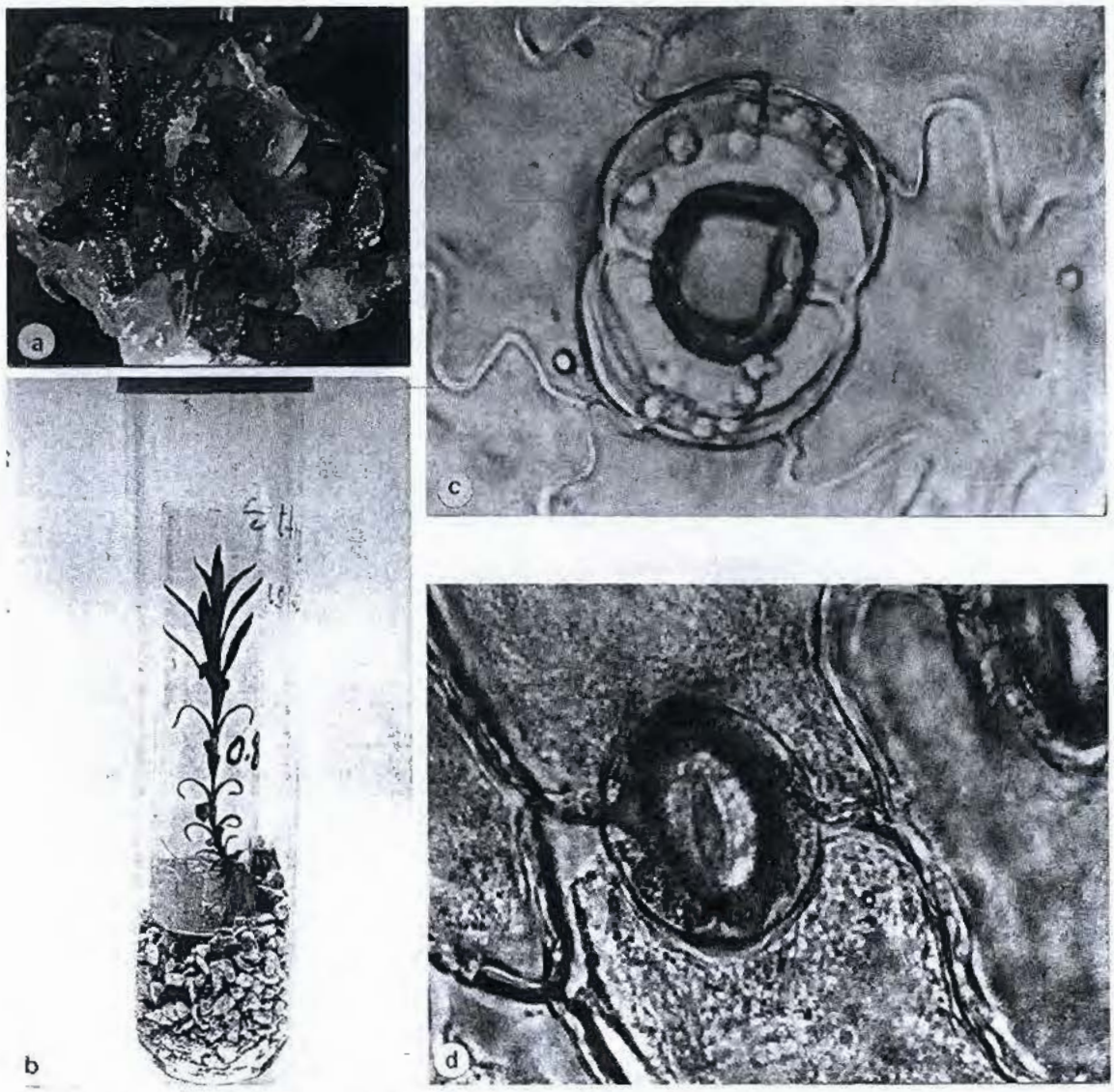

FIG. 1. Succulent carnation plant (*a*) cultured on 0.8% agar medium and a normal plant (*b*) in an uncapped tube transferred after 14 days to a desiccant. *Note* upper normal leaves. An open stoma from a succulent leaf (*c*) and a closed stoma from a normal leaf (*d*) after 6 h in the dark (53,58,59).

and the high deposition of callose in the malformed guard cells of vitreous carnation leaves (1,55,57).

Thin cell walls, large intercellular air spaces, and reduced vascular tissue were observed also in vitreous stems which were smaller in diameter, lacked sclerenchymatous tissue, and the cortical and pith cells were hyperhydrated (47,48). In vitreous carnation plants procambial strands were not observed, and the vascular bundles lacked normal organization (29).

Modified Shoot Physiology

Metabolic and physiologic processes associated with photosynthesis and transpiration are severely influenced by abnormal leaf morphology. The culture environment can effect and lead to changes in enzymatic activity, resulting in altered plant metabolic processes. Some of the responses often resemble plants under stress conditions.

Leaf hyperhydration has been attributed to defective lignification, reduced cellulose synthesis, changes in cell wall extensibility, and altered cell water relations. These can lead to reduced cell turgor pressure, changes in the water potential, and increased water uptake and as a result, to tissue hyperhydration (16). Cultured shoots or apices, once excised and planted in vitro, are exposed to an aeration stress, either by being submerged in the liquid medium, in the condensation water or by being exposed to high relative humidity (90 to 100%) in the sealed cultures. These conditions, which may be analogous to waterlogging, a stress known to cause excessive C_2H_4 evolution (52), may explain the high levels of ethylene evolution by vitreous plants (13,20,22,24,57). Hypolignification was attributed to lower levels of phenols and the inadequate activity of the enzyme hydroxy cinnamic CoA-ligase (39). Hypolignification was also related to a decrease in phenylalanine ammonia lyase (PAL) activity. Various inhibitors of ethylene synthesis did not pre-

reported to be associated with abnormal morphogenesis in vitro (53). These factors control the physical and chemical state of the medium and the culture atmosphere, mainly H_2O vapor, CO_2, and C_2H_4 levels.

Media in general are either liquid or semisolid, the latter is achieved by the addition of various concentrations of gelling agents, the most common of which is agar (7,38). Agar contributes to the matrix potential, the relative humidity, and affects the availability of water and dissolved substances from the medium in the sealed containers (7,9). Vitrification was observed in several species when the shoot system was cultured in liquid or low agar, semisolid medium (55). Increasing the agar concentration reduced vitrification but very often also lowered the propagation ratio (9,20,49,58) because it affected the availability of various components in the medium, in particular cytokinins (7).

The relative humidity (RH), resulting from temperature gradients in the container and the medium's water status, plays a major role in vitrification (8). The differences appear early in ontogeny and are nonreversible once initiated (58). Reduction of the RH in the culture atmosphere through use of desiccants, increased wax deposition in carnation (53) and cabbage plants (45). Application of a lanolin layer over the agar medium reduced the RH in the cultures to 35%, causing shoot desiccation in chrysanthemums and increasing wax deposition in cabbage (50). In apple, exposure to low RH improved plant survival ex vitro by improving normal leaf development (5). According to Maene and Debergh (33) and Vanderschaeghe and Debergh (46), reduction in RH can be achieved by placing the cultures on a cooled plate, which causes the water vapor to condense on the agar medium and thus reduce vitrification.

Various constituents in the medium in addition to gelling agents regulate the water potential and may affect plant morphogenesis (32,41,58). In African violets, increasing the agar concentration to 1.2% induced normal leaf development (Fig. 2 *a, b*). Overcoming the effect of high agar levels on proliferation can be achieved by the subculture from a proliferating (liquid) to a hardening (agar) medium (Fig. 3) or by a two-phase culture to which a hardening medium is added after the proliferation stage (Ziv, unpublished). The main problem is to control leaf development after leaf primordia or new buds have formed, because once the leaves attain even a minimal size, the deformations are non-reversible (58).

Of the various minerals constituting the most commonly used Murashige & Skoog medium, NH_4^+ and Ca^{++} were the two major elements reported to be involved in vitrification of several species. Using media with lower levels of minerals, or only half of the MS salts, improved carnation and cucumber plant development (56,59). Reducing the level of NH_4^+ in the medium increased lignification and reduced vitrification in willow, plum, and in cacti species (2,6,28). In carnation, lowering the NH_4^+ to NO_3^- ratio improved plant morphogenesis (57). In sour cherry, no correlation was found between Ca^{++} accumulation and vitreous leaves, which showed higher acid phosphatase activity (4). Elevated Ca^{++} was found to reduce vitrification in woody species (27). There is however some conflicting evidence that elevated Ca^{++} may interfere with lignification through activation of peroxidases and induction of callose formation (16,21,23,57). Reducing Ca^{++} to 1 m*M* induced an increase in chlorophyll and wax content in carnation leaves (Table 1). Vitreous carnation plants lost Ca^{++} and Mg^{++} to the medium while taking up K^+ from the liquid medium (25). Vitrification was attributed by Kevers and Gaspar (25) to an osmotic shock in the liquid medium, through the effect of a direct contact between the tissue and the components of either liquid or semisolid medium.

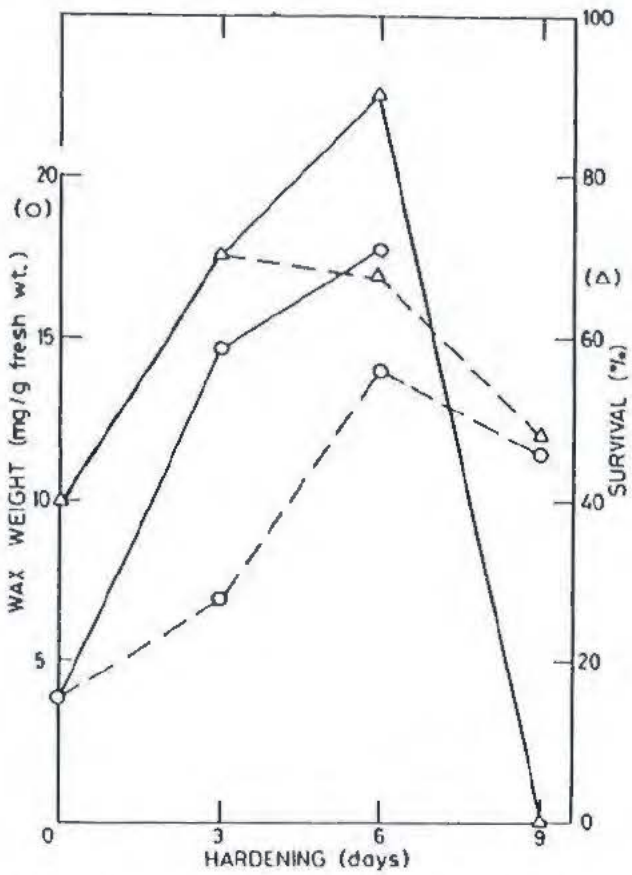

FIG. 4. Effect of hardening carnation plants in vitro under 60% RH and 80 or 160 $\mu E \cdot m^{-2} \cdot s^{-1}$ on leaf wax content and survival ex vitro. Wax was not determined in desiccated plants cultured for 9 days under 160 $\mu E \cdot m^{-2} \cdot s^{-1}$ (after 13). *Broken line* 80; *solid line* 160 $\mu E \cdot m^{-2} \cdot s^{-1}$.

The most commonly used growth regulators are auxins and cytokinins supplied either singly or in various combinations, depending on the species and the type of explant. Some of the evidence presented on the effects of growth regulators on abnormal morphogenesis is limited and contradictory. Leshem (30) suggested that an imbalance in auxin and cytokinin levels induced vitrification in carnation, whereas lowering the level of indole acetic acid (IAA) in conifers reduced vitrification when coupled with reduced kinetin (12).

Reports on the involvement of cytokinins in vitrification indicate that high levels of cytokinins in liquid, or semisolid media with a high water potential, induced abnormal shoot morphogenesis (7,49). In several species, reducing the cytokinin levels decreased the extent of vitrification (12,16,31,61).

Ethylene is a growth regulator found to be involved in vitrification because it was directly related to the physical and chemical condition in the culture. Increased ethylene evolution under various culture conditions was reported in several species. In carnation it was most pronounced in tightly sealed liquid cultures (13,20,22, 24,35).

Ethylene accumulation was related to hypolignification and to

changes in cell wall elastic properties. These changes induced hyperhydration due to reduction in turgor pressure and an increase in water uptake by the tissue (25,39).

Loosely capped cultures provide proper aeration and allow the removal of C_2H_4 and excess water vapor, conditions that were found to decrease vitrification (13,35). As can be seen in Table 1, various culture factors improved chlorophyll and wax content in carnation leaves. The level of agar in the medium, the RH, and the addition of 10^{-5} *M* AOA induced the highest increase in the production of chlorophyll and cuticular wax.

The carnation plant has been used for over 10 yr in our laboratory as an experimental plant system for studies on in vitro plant development and hardening before transplanting and on the survival of the plants ex vitro. Carnation shoot apices cultured as previously described (13,58,59) were hardened in vitro for up to 9 days under 60% RH and two photon flux densities. As can be seen in Fig. 4, hardening for 6 days under 160 $\mu E \cdot m^{-2} \cdot s^{-1}$ induced the highest level of waxes in the leaves, and 92% of the plants survived transplanting. Hardening for 9 days caused severe plant desiccation. Hardening under 80 $\mu E \cdot m^{-2} \cdot s^{-1}$ induced a lower wax content after 6 days under 60% RH, and plant survival decreased to about 70%. A further decrease in wax content and survival was observed after 9 days of hardening under these conditions (13).

In conclusion, it is now clear that abnormal morphogenesis in vitro is a consequence of physiologic disorders brought about through changes in various metabolic pathways (16,55). These physiologic disorders induce the development of abnormal plants that cannot survive transplanting and require a gradual transition stage. If acclimatization is carried out in vitro, before transplanting, the existing leaves that developed under such conditions will survive ex vitro. The control of the physical state of the medium, the level of minerals, growth regulators, and the composition of the atmosphere in the containers (55) can provide the necessary conditions for normal leaf development. An additional measure reported recently (40,54,55,60) was the use of growth retardants, such as paclobutrazol and ancymidol, to harden leaves before transplanting. Thus, in vitro acclimatization of plants in stage III will improve plant survival and reduce the need for extended acclimatization in vivo.

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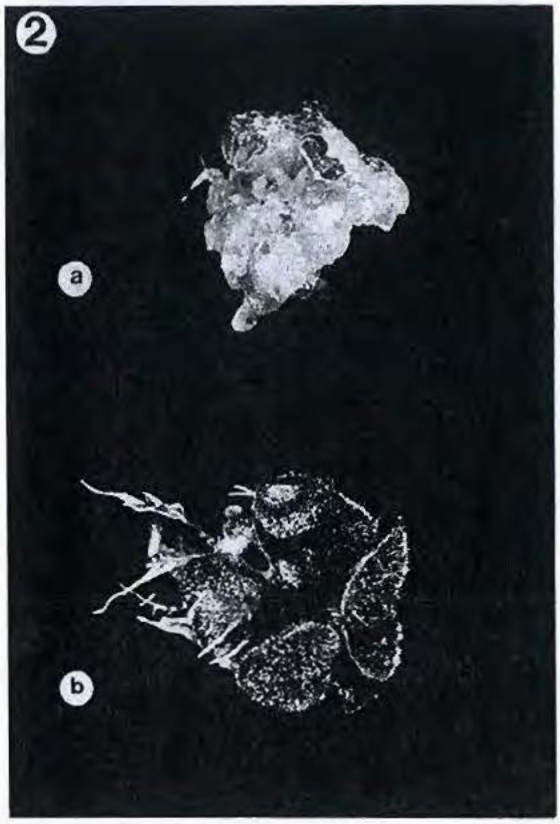

FIG. 2. Vitreous African violet plants cultured in liquid medium (*a*) and on 1.2% agar medium (*b*).
FIG. 3. Carnation plant cultured in liquid medium (succulent leaves) and transferred after 18 days to 1.2% agar medium (normal leaves).

vent vitrification in carnation (24), with the exception of one cultivar, "Ceris Royallete." Addition of 10^{-5} *M* amino-oxyacetic-acid (AOA), an inhibitor of ethylene formation to the medium, decreased leaf hydration and increased chlorophyll and wax levels in liquid-cultured plants (Table 1) (13).

The lower metabolic activity associated with photosynthesis and carbohydrate assimilation causes in vitro plants to become heterotrophic. Grout and Donkin (19) studied the photosynthetic apparatus and activity in cauliflower meristem cultures. Although adequate as far as the electron transport system, the leaves had lower chlorophyll and reduced ribulose bisphosphate carboxylase activity, resulting in poor carbon assimilation.

TABLE 1

THE EFFECT OF CULTURE CONDITION ON CHLOROPHYLL AND WAX CONTENT IN CARNATION LEAVES AFTER 4–6 WEEKS IN CULTURE (AFTER 13, 53, 58 ZIV, UNPUBLISHED)

Culture Condition	Chlorophyll, μg/mg FW[a]	Wax, mg/g FW[a]
Basal medium[b]	0.21 ± 0.2	3.9 ± 0.6
1/2 MS minerals	0.52 ± 0.3	8.9 ± 0.9
NH_4^+ 10 m*M*	0.87 ± 0.9	12.4 ± 1.1
Ca^{++} 1.0 m*M*	0.64 ± 0.5	15.1 ± 1.2
Agar 1.2%	1.29 ± 1.4	28.6 ± 1.9
ABA 10^{-4} *M*	0.46 ± 0.2	7.1 ± 0.8
AOA 10^{-5} *M*	1.13 ± 1.3	27.4 ± 3.1
RH 60%	0.97 ± 1.1	21.6 ± 1.9

[a] FW = Fresh weight.
[b] Carnation apices cultured on MS 0.8% agar medium (NH_4^+ 20 mM Ca^{++} 3 mM, RH 100%) with 2.3 μ*M* KIN and 2.7 μ*M* NAA. All other treatments are changes made in the basal medium.

Calcium is known to regulate polymerization of tubulin in the microtubuli and is associated with β-1,3-glucan synthase activity and in promoting callose formation (11,21). It is possible that in addition to the effect of Ca^{++} on the release from the cell walls of peroxidases, enzymes that were correlated with hypolignification (22), Ca^{++} availability caused microfibril disorientation and callose deposition in the mesophyll as well as in the epidermal and guard cells. In carnation the failure of the guard cells to close in response to ABA, was due to a deformed cell wall. Although the osmotic potential of the guard cells increased in response to ABA, indicating that the protoplast of the nonfunctional stomata responded to the closing signal, the pore failed to close (59). Thus, hypolignification, microfibril disorientation, and deposition of callose instead of cellulose could have contributed to tissue hyperhydration, stomatal failure, and the phenomenon of vitrification.

The Control of Shoot Development by Culture Condition Manipulation

The culture environment, organic and inorganic components, growth regulators, and light and temperature are the major factors

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3. LISTAS ASISTENCIA ACTIVIDADES DE DIFUSION

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FECHA 25/05/2005

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