Plant Pathology (1994) **43**, 998–1008

Detection of the mycoplasma-like organism associated with lethal yellowing disease of palms in Florida by polymerase chain reaction

N. A. HARRISON, P. A. RICHARDSON, J. B. KRAMER *and* J. H. TSAI
University of Florida, IFAS, Fort Lauderdale Research and Education Center, 3205 College Ave., Fort Lauderdale, Florida 33314, USA

DNA amplification by polymerase chain reaction (PCR) was used specifically to detect the mycoplasma-like organism (MLO) associated with lethal yellowing disease of palms in Florida. For PCR, a pair of oligonucleotide primers was synthesized according to partial sequences of a cloned 1·3 kbp fragment of lethal yellowing MLO-specific genomic DNA isolated from a diseased windmill palm (*Trachycarpus fortunei*). A DNA product of about 1 kbp was specifically amplified by PCR in reaction mixtures containing template DNA derived from either heart, inflorescence or leaf tissues of lethal yellowing-affected palms. PCR performed for 35 cycles with as little as 5 pg of DNA template, in some instances, was sufficient consistently to amplify the same lethal yellowing MLO DNA product from hearts of 11 species comprising 30 symptomatic palms. Similar reliable and reproducible detection of the lethal yellowing MLO in palm inflorescence spikelets was also achieved after 35 cycles of PCR. When template DNA for PCR was derived from tissues of the the most immature emerging leaf, a 40-cycle reaction was sufficient for consistent foliar detection of the pathogen in all coconut palms including palms with earliest visible symptoms of disease.

INTRODUCTION

Lethal yellowing disease of the coconut palm (*Cocos nucifera*) has been endemic to parts of the western Caribbean region for at least 100 years (Howard, 1983). During the last three decades, epiphytotics of lethal yellowing prompted by unknown factors have killed millions of susceptible Tall-type coconut palms in both Jamaica (Anonymous, 1986) and southern Florida (McCoy *et al.*, 1983). Today, the once prevalent Jamaica Tall cultivar has been virtually eliminated from both geographic localities.

In areas of southeast Florida severely affected by coconut lethal yellowing, lethal decline diseases of numerous other palm species were also recognized (Thomas, 1979). As with coconut lethal yellowing, mycoplasma-like organisms (MLOs) were implicated as the probable aetiological agents of these additional diseases. This evidence, coupled with the appearance on some palm species of symptoms superficially similar to those of coconut lethal yellowing, indicated that these diseases were probably all caused by the same pathogen (McCoy *et al.*, 1983). The subsequent transmission of lethal yellowing to coconut, Manila (*Veitchia merrillii*) and Thurston (*Pritchardia thurstonii*) palms by field-collected American palm cixiids (*Myndus crudus*), the suspected principal vector of lethal yellowing in the Americas (Howard *et al.*, 1983), strongly supported this assertion.

Further evidence of the involvement of the lethal yellowing MLO in lethal declines of other palm species was recently obtained with DNA hybridizations using cloned genomic DNA fragments of the pathogen as probes (Harrison *et al.*, 1992). Reliable MLO detection was achieved when DNA samples extracted from immature palm leaf bases (heart tissues) rich in functional phloem were probed. However, most mature organs of infected palms contain few MLOs (Thomas & Norris, 1980). In this respect, lethal yellowing resembles MLO-associated diseases of other woody perennial plant hosts (Hibben *et al.*, 1991; Sinclair *et al.*, 1992). Consistent detection of MLO infections in palms required the use of ^{32}P-labelled DNA probes to achieve the necessary detection sensitivity (Harrison *et al.*, 1992, 1994). This prerequisite has limited the utility of DNA probes for investigations of lethal yellowing elsewhere in the western Caribbean where the disease is presently most active.

Selective enzymatic amplification of MLO DNA from mixtures with host DNA by polymerase chain reactions (PCR) is a recent approach to studies on MLOs. PCR amplification of MLO 16S ribosomal RNA (rRNA) gene sequences and restriction site analysis (Ahrens & Seemüller, 1992; Lee *et al.*, 1993b; Schneider *et al.*, 1993) or cycle sequencing (Namba *et al.* 1993; Schneider *et al.*, 1993) of the rDNA products have been used to differentiate and group MLOs for taxonomic purposes. MLO-specific PCR has also been developed by exploiting anonymous sequences of cloned MLO DNA as primers (Deng & Hiruki, 1991a; Schaff *et al.*, 1992; Lee *et al.*, 1993a). Furthermore, the sensitivity of detection achievable by MLO-specific PCR substantially exceeds the lower limits reported for DNA probe hybridizations (Schaff *et al.*, 1992) and is sufficient to monitor MLO acquisition by leafhoppers during feeding on infected plants (Vega *et al.*, 1993). Thus, PCR-based techniques could greatly enhance detection of MLOs normally present only in low titres, such as the palm lethal yellowing agent, and possibly facilitate detailed investigations of lethal yellowing disease epidemiology.

We report the development of primers from a genomic DNA sequence of the lethal yellowing MLO isolated from a windmill palm (*Trachycarpus fortunei*) which enable specific detection of the pathogen. The sensitivity and reliability of the lethal yellowing-specific PCR for detecting the pathogen in various tissues of selected palm species was evaluated.

METHODS

Sources of healthy and diseased palms

Heart tissues consisting mostly of immature leaf bases were obtained from 30 naturally infected 7–12-year-old palms showing mid-stage (yellowing phase) lethal yellowing symptoms (McCoy *et al.*, 1983). With the exception of Manila palms (*V. merrillii*), all others were located on the grounds of the University of Florida's Fort Lauderdale Research and Education Center (FLREC). Palms were felled and samples collected from representatives of the following 10 species: dwarf sugar palm (*Arenga engleri*), giant fishtail palm (*Caryota rumphiana*), *Cocos nucifera* (cultivars Hawaiian Tall, Jamaica Tall, Malayan Dwarf, Panama Tall) and Maypan hybrid coconut (Malayan Dwarf × Panama Tall), hurricane palm (*Dictyosperma album*), spindle palm (*Hyophorbe verschafeltii*), Chinese fan palm (*Livistona chinensis*), footstool palm (*L. rotundifolia*), true date palm (*Phoenix dactylifera*), silver date palm (*P. sylvestris*), and windmill palm (*T. fortunei*). Five diseased Manila palms each of an undetermined age were provided to us by local homeowners. Young, 1–2-year-old container-grown palms in shadehouses served as sources of healthy tissues for comparison.

Single immature (unemerged) inflorescences with partially necrotic spikelets were removed from solitary, bearing coconut (cultivar Jamaica Tall) palms growing within the vicinity of Miami and Naples, Florida. As both palms also displayed premature nutfall, these combined symptoms were indicative of early-stage (primary phase) lethal yellowing symptoms (McCoy *et al.*, 1983). Similar inflorescences were removed from five symptomatic coconut (cultivar Malayan Dwarf) palms and a Chinese fan palm at the FLREC. Immature, unblemished coconut inflorescences were also excised from a symptomless Malayan Dwarf and an Indian Green Dwarf cultivar for use as sources of apparently healthy tissues.

Non-necrotic leaflets retaining green colour were removed from proximal portions of all leaves of two non-bearing coconut (cultivar Panama Tall) palms with early lethal yellowing foliar symptoms. Leaflets were also sampled from lower portions of the youngest, emerging leaf (spear) on five additional coconut palms. These included two mature, bearing Malayan Dwarfs and a non-bearing Hawaiian Tall with early yellowing phase symptoms; a bearing Malayan Dwarf with primary symptoms; and a non-bearing Jamaica Tall with spear necrosis only.

Other MLO-associated plant diseases

Plants affected by various other MLO-associated diseases indigenous to Florida were maintained in shadehouses. These included sweet corn (*Zea mays* 'saccharata' cv. Aristogold Guardian) with maize bushy stunt (Davis *et al.*, 1988), Madagascar periwinkle (*Catharanthus roseus*) singly infected with pigeon pea witches' broom (Harrison *et al.*, 1991), or periwinkle witches' broom (McCoy & Thomas, 1980), or periwinkle virescence disease. Periwinkle infected with the following additional MLOs were also kindly provided by other researchers: eastern aster yellows, J. A. Wyman (University of Wisconsin, Madison, WI, USA); western X, peach yellow leafroll strain and prune strain, B. C. Kirkpatrick

(University of California, Davis, CA, USA); Yucatan periwinkle virescence, M. A. Villanueva, (Centro de Investigación Cientifica de Yucatán, A.C., Merida, Mexico). Coconut inflorescence DNAs extracted from an East African Tall palm in Tanzania with lethal disease, and a West African Tall palm in Nigeria with Awka disease, were both kindly provided by P. Jones (Rothamsted Experimental Station, Harpenden, UK).

Culturable mollicutes

Spiroplasma kunkelii Florida isolate T80 (Davis *et al.*, 1984); *S. citri* California isolate 189 (McCoy *et al.*, 1981); and the *Cocos* spiroplasma isolate N525 (Eden-Green & Waters, 1981), kindly provided by R. E. Whitcomb (USDA ARS, Beltsville, MD, USA), were cultured in C3-G medium (Liao & Chen, 1977). *Acholeplasma axanthum* (S743), *A. oculi* (19L), both NIAID reference strains, and an unidentified *Acholeplasma* sp. (J233), originally from a lethal yellowing-affected coconut palm (Eden-Green & Tully, 1979) were kindly provided by J. G. Tully (Frederick Cancer Facility, Frederick, MD, USA) and cultured in SP-4 medium (Tully *et al.* 1977).

DNA extractions

Preparations enriched with MLOs were obtained from symptomatic plant tissues by differential centrifugation after tissues were ground in an osmotically augmented buffer (Harrison *et al.* 1991, 1992). Total DNAs were extracted from these preparations as previously described (Harrison *et al.*, 1992). DNAs extracted from comparable tissues of seed-grown or symptomless landscape plants served as experimental controls. Cells from spiroplasma and acholeplasma cultures were collected by centrifugation at 20 000 *g* for 30 min at 4°C and extracted by the procedure of Dellaporta *et al.* (1983).

Small-scale (3 g) extractions of total DNA from freshly harvested coconut leaflet laminae were prepared by the procedure of Doyle & Doyle (1990) except that the DNA extraction buffer also contained 10 g/l PVP-40. All DNA extracts were quantified by fluorometry (TKO-100 minifluorometer, Hoefer Scientific Instruments, San Francisco, CA) and stored at 4°C.

DNA manipulations

Lethal yellowing MLO DNA for molecular cloning was obtained by repeated caesium chloride–bisbenzimide buoyant density gradient centrifugation (Kollar *et al.*, 1990) of total DNAs extracted from heart tissues of a lethal yellowing-diseased windmill palm. About 400 ng of the gradient-enriched lethal yellowing MLO DNA was partially digested with *Hin*dIII (Promega Biological Research Products, Madison, WI, USA) for 1 h at 37°C. Resulting fragments were ligated with dephosphorylated *pUC*19 using a 10 : 1 insert:vector ratio and cloned in *Escherichia coli* DH5-α cells (Gibco BRL Life Technologies, Gaithersburg, MD, USA). Recombinant plasmid DNA was extracted by alkaline lysis from small-scale Luria–Bertani (LB) broth cultures of selected recombinant colonies as described by Sambrook *et al.* (1989). About 200 ng of each DNA preparation was blotted onto nylon membranes (Nytran, Schleicher and Schuell Inc., Keene, NH, USA) following the protocol of Lee & Davis (1988). Replicate blots were air dried and then baked at 80°C for 30 min prior to hybridizations.

Recombinant plasmids containing lethal yellowing MLO DNA inserts were tentatively identified by moderately stringent differential dot hybridizations using *Hin*dIII-digested healthy coconut palm DNA or gradient-enriched lethal yellowing MLO DNA as probes. Blots were hybridized with probes using previously reported conditions (Harrison *et al.*, 1992) then sealed in plastic wrap and exposed to Konica PB7, X-ray film (Konica Medical Corp., Wayne, NJ, USA) with an intensifier screen (Lightning Plus, DuPont, Newark, DE, USA) for 5 days at −75°C.

To identify pathogen-specific MLO DNA inserts, blots of recombinant plasmid DNA were stripped of initial probes by boiling each membrane in 0·1 × SSC, 5 mg/l SDS (Sambrook *et al.*, 1989) and then reprobed at moderate stringency with [^{32}P] dATP labelled, gradient-enriched DNA of the western X (prune strain) MLO or with total DNAs extracted from healthy periwinkle. Blots were stripped again and reprobed with gradient-enriched DNA of the pigeon pea witches' broom MLO.

Cloned DNA inserts from eight recombinant plasmids which hybridized only to enriched lethal yellowing MLO DNA were labelled with [^{32}P] dATP by using random primers (random primed DNA labelling kit, Boehringer Mannheim Biochemicals, Indianapolis, IN, USA). Inserts were used individually to probe dot blots of various undigested plant and mollicute

DNAs and Southern blots of *Hin*dIII-digested healthy plant DNAs or DNAs from plants with various MLO-associated diseases. For dot blots, each sample was applied to nylon membranes as a series of twofold dilutions beginning with 2 μg. For Southern blots, 1 μg of each sample DNA was digested with *Hin*dIII for a minimum of 4 h at 37 C and electrophoresed in 7·5 mg/ml agarose (Low EEO grade, Fisher Scientific, Pittsburgh, PA, USA) gels using 1 × TAE (40 mM Tris-acetate, 1 mM EDTA) as running buffer. DNA was blotted from gels onto nylon membranes by a modification of Southern's method (Sambrook *et al*., 1989).

Oligonucleotide primers and PCR conditions

Probe LYTC24, a 4.5 kbp fragment of LY MLO DNA, which hybridized only with DNA of lethal yellowing-diseased palms, was digested with *Xba*I. One of the two resulting subfragments (TC24-A, 1·3 kbp) was ligated with *Hin*dIII–*Xba*I-digested *pUC*19 and cloned, as before. Partial sequencing of subclone TC24-A was achieved by using M13/*pUC* primers and standard dideoxy nucleotide termination reactions (Sambrook *et al*., 1989). A pair of oligonucleotide primers was synthesized on the basis of the sequence data. Both sequencing and primer synthesis were performed by the DNA sequencing and synthesis Core laboratories at the University of Florida's Interdisciplinary Center for Biotechnology Research.

For PCR, sample DNAs for use as template were diluted to 25 ng/μl with sterile distilled water. Amplifications were performed in 50 μl final reaction volumes each containing 50 ng of sample DNA template, 50 ng of each primer, 125 μM of each dNTP, 1·5 U of AmpliTaq DNA polymerase (AS) with recommended PCR buffer (Perkin Elmer Cetus, Norwalk, CT, USA) and overlaid with mineral oil. PCR was performed for 35 or 40 cycles in a thermal block cycler (Model 110S, Coy Laboratory Products Inc., Ann Arbor, MI, USA) using the following parameters: 30 s (90 s for first cycle) denaturation step at 94°C, annealing at 53°C for 50 s and primer extension at 72°C for 80 s. Reaction mixtures containing healthy plant DNA or sterile distilled water substituted for template DNA served as negative controls in each experiment. Following all amplifiications, 10 μl of each reaction mixture was analysed by electrophoresis in a 10 mg/ml agarose gel. PCR products in gels were stained with ethidium bromide, visualized by UV transillumination and photographed.

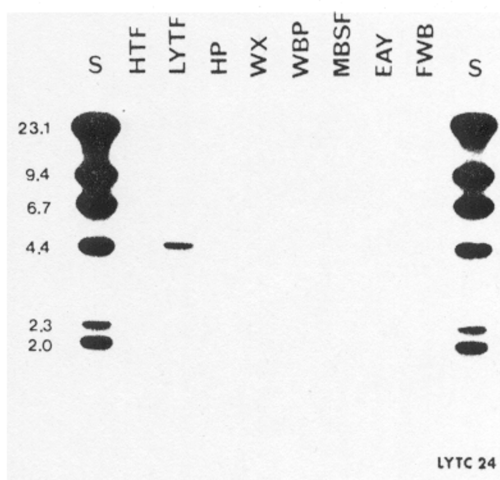

Fig. 1. Southern blot hybridization of probe LYTC24, a 4·5 kbp chromosomal fragment of the palm lethal yellowing (LY) mycoplasmalike organism (MLO) to *Hin*dIII-digested DNA derived from healthy plants and plants with various MLO-associated diseases. DNA from: HTF, healthy *Trachycarpus fortunei* (windmill palm); LYTF, LY-diseased windmill palm; HP, healthy *Catharanthus roseus* (periwinkle); WX, periwinkle infected with western X (prune strain); WBP, periwinkle with pigeon pea witches' broom; MBSF, sweet corn (*Zea mays* 'saccharata' cv. Aristogold Guardian) with Florida maize bushy stunt; EAY, periwinkle with eastern aster yellows; FWB, Florida periwinkle witches' broom. S, lambda DNA/*Hin*dIII fragments.

RESULTS

Of the 94 recombinant plasmids initially evaluated by differential dot hybridizations, 79 were judged to contain cloned MLO DNA inserts. Forty-two of these subsequently hybridized with probes consisting of either gradient-enriched western X MLO or pigeon pea witches' broom MLO DNAs, indicating inserts to be MLO-specific but not lethal yellowing MLO-specific (data not shown). Cloned inserts, ranging in size from 0·8 to 4·5 kbp, were excised from eight of the remaining 37 recombinant plasmids and used as probes to confirm their disease specificity. One of the these, clone LYTC24, consisting of a 4·5 kbp fragment of lethal yellowing MLO chromosomal DNA, detected DNA derived only from lethal yellowing-affected palms during both moderately stringent dot (data not shown) and Southern (Fig. 1) hybridizations.

Signals resulting from dot hybridizations between probe LYTC24 and DNA derived

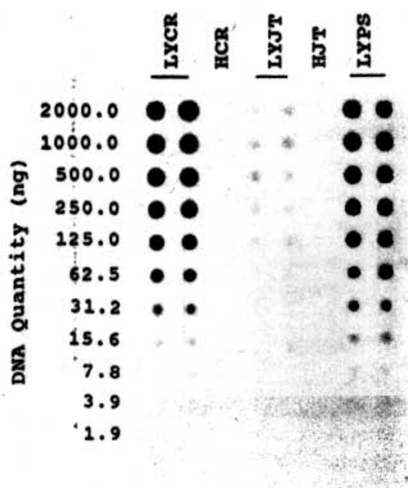

Fig. 2. Dot hybridization of probe LYTC24 (4·5 kbp) to DNA extracted from heart tissues of three palm species with mid-stage foliar symptoms indicative of lethal yellowing (LY) disease. LYCR, *Caryota rumphiana* (giant fishtail palm); HCR, healthy *C. rumphiana*; LYJT, *Cocos nucifera* (coconut palm cultivar Jamaica Tall); HJT, healthy *C. nucifera* cultivar Jamaica Tall; LYPS, *Phoenix sylvestris* (silver date palm).

palms differed in intensity according to the particular palm species. Signals observed for ornamental palm species *C. rumphiana* and *P. sylvestris*, were at least 10- to 12-fold stronger than those routinely encountered for Tall-type *C. nucifera* cultivars. Representative hybridization signals are illustrated in Fig. 2. Following both dot and Southern hybridizations, a minimum of 115 h of autoradiography was necessary in order to resolve clearly probe signals. These weak hybridizations indicated that probe LYTC24 hybridized to a low copy genomic MLO DNA sequence, or reflected an overall low concentration of MLO in lethal yellowing-diseased palms, or both.

Restriction site analysis of probe LYTC24 revealed a single internal *Xba*I site yielding two fragments of about 1·3 kbp and 3·2 kbp, respectively. Partial enzymatic sequencing of the smaller subcloned fragment, LYTC24-A, yielded sequence data with an overall A + T composition of about 82% (Fig. 3). Two 25-mer oligonuclotide sequences designated LYF-1 and LYR-1, with an estimated T_m of 58·3 and 58·7°C, respectively, were synthesized on the basis of the

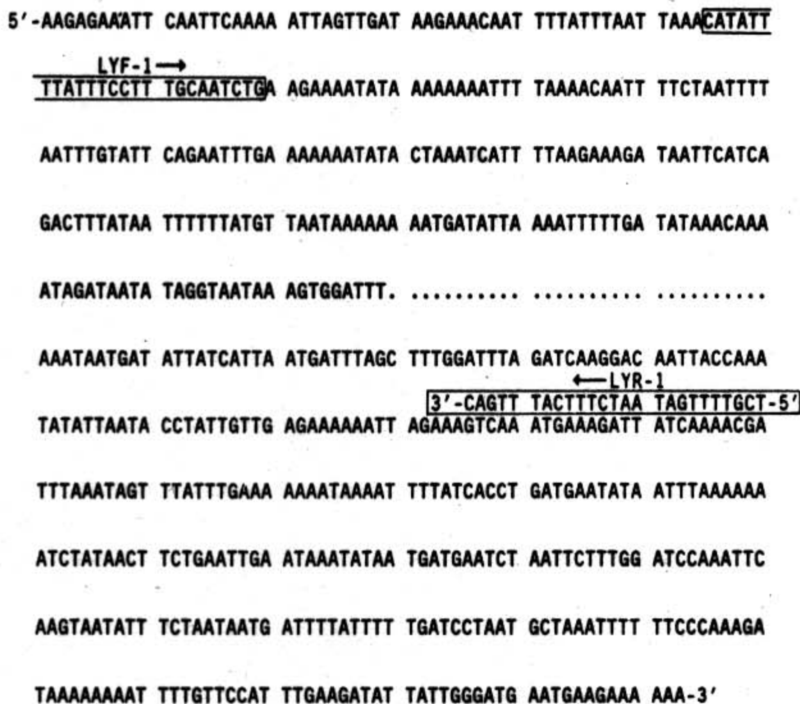

Fig. 3. Partial sequence of subclone LYTC24-A, a 1·3-kbp *Hin*dIII–*Xba*I fragment of lethal yellowing (LY) mycoplasma-like organism chromosomal DNA. The position of primers LYF-1 and LYR-1 used for polymerase chain reactions are indicated in boxes.

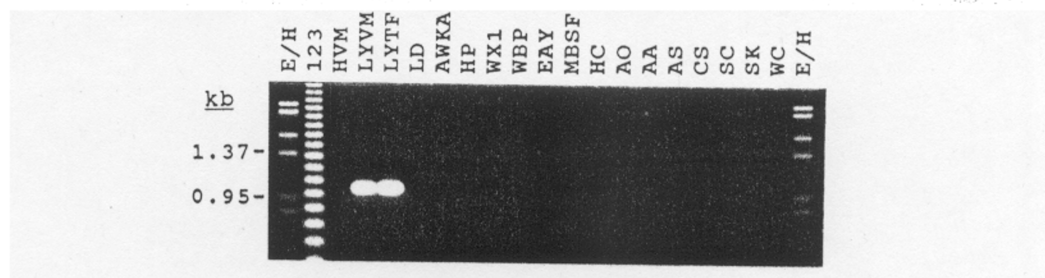

Fig. 4. Agarose gel electrophoresis of PCR products from healthy plants, plants with various mycoplasma-like organism (MLO)-associated diseases and from culturable mollicutes. PCR products are a result of a 35-cycle reaction using a primer pair derived from a cloned 1·3 kbp fragment of LY (LY) MLO chromosomal DNA. PCR template DNAs were derived from: HVM, healthy *Veitchia merrillii* (Manila palm); LYVM, LY-diseased Manila palm; LYTF, LY-diseased *Trachycarpus fortunei* (windmill palm); LD, lethal disease-affected *Cocos hucifera* cultivar East African Tall from Tanzania; AWKA, Awka disease-affected *C. nucifera* cultivar West African Tall from Nigeria; HP, healthy *Catharanthus roseus* (periwinkle); WX1, periwinkle infected with western X (peach yellow leafroll strain); WBP, periwinkle with pigeon pea witches' broom; EAY, periwinkle with eastern aster yellows; MBSF, sweet corn (*Zea mays* 'saccharata' cv. Aristogold Guardian) with Florida maize bushy stunt; HC, healthy corn; AO, *Acholeplasma oculi*; AA, *A. axanthum*; AS, an uncharacterized *Acholeplasma* sp. (J233) from coconut palm; CS, *Cocos* spiroplasma; SC, *Spiroplasma citri*; SK, *Spiroplasma kunkelii*. WC, water control; E/H, lambda DNA *Eco*RI–*Hin*dIII fragments; 123, BRL 123 bp ladder.

sequence data. When used during initial PCR, this primer pair permitted amplification of a prominent DNA product of about 1 kbp from reaction mixtures containing template DNA derived from heart tissues of both lethal yellowing-diseased Manila and windmill palms (Fig. 4). The size of the DNA product was consistent with its predicted size according to the distance between primer sequences within the original lethal yellowing MLO chromosomal DNA fragment. No product was amplified by PCR from similar mixtures containing template DNA extracted from either healthy Manila palm or from coconut palms with African MLO-associated lethal decline diseases. No DNA product was evident either when template DNA was derived from various other MLO-associated diseases or from culturable acholeplasmas and spiroplasmas.

The reliability of the chosen primer sequences to amplify lethal yellowing MLO DNA was established by PCR analysis of template DNAs from heart tissues of 30 different symptomatic palms. After 35 cycles, amplification of a 1 kbp DNA product was again repeatedly achieved from all palm specimens, which included 14 individuals and five cultivars of coconut palm (Fig. 5A) as well as 10 additional ornamental species comprising 16 palms (Fig. 5B). Amplifications of a similar size product were also obtained when the template for PCR consisted of DNA from immature, partially necrotic inflorescences of palms exhibiting either primary or early yellowing phase lethal yellowing symptoms (Fig. 5C). No PCR products were amplified from DNAs of healthy palm hearts or from inflorescence DNAs of other symptomless coconut palms.

Use of decreasing quantities of DNA extracted from hearts of lethal yellowing-diseased giant fishtail, coconut cultivar Jamaica Tall, and windmill palm for PCR provided a measure of the relative sensitivity of this technique for detection of the lethal yellowing MLO in different palm hosts. After 35 cycles of PCR and agarose gel electrophoresis of 10 μl of each final reaction mixture, amplification of MLO DNA was clearly evident in mixtures which initially contained as little as 5 pg of DNA from each of the three palm species (Fig. 6).

A 40-cycle reaction was required to detect lethal yellowing MLO DNA in 12 of 35 leaflet DNA samples derived from seven symptomatic coconut palms. In two non-bearing coconut palms (cultivar Panama Tall) examined in detail (Fig. 7A, B), MLOs were undetectable in basal leaflets from all but the youngest leaves. When sampling was limited to the spear leaf only, positive detection of the pathogen was achieved for all five additional palms tested (Fig. 7B).

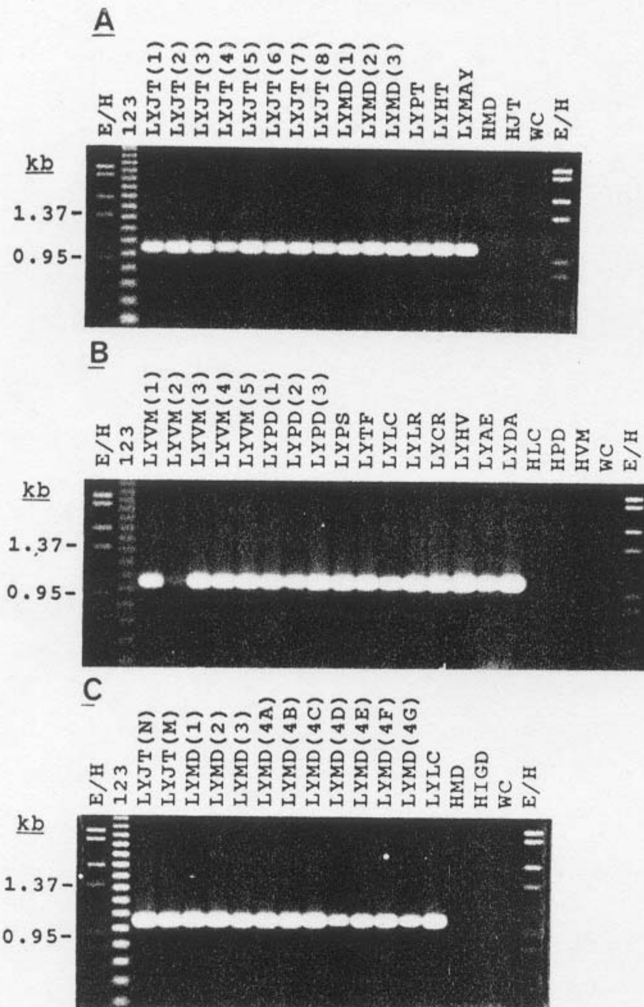

Fig. 5. PCR analysis of DNA extracted from tissues of healthy container- grown palms, symptomless landscape palms and landscape palms with mid-stage foliar symptoms indicative of lethal yellowing (LY) disease. After 35 cycles of amplification, one-fifth of each reaction mixture was analysed by 10 mg ml agarose gel electrophoresis. Template DNA for PCR was derived from heart tissues of healthy and LY-symptomatic *Cocos nucifera* (coconut palm) cultivars (A), or heart tissues of other palm species (B), or immature palm inflorescences (C). (A) LY-diseased *C. nucifera* cultivars: LYJT (1-8), eight Jamaica Tall palms; LYMD (1-3), three Malayan Dwarf palms; LYPT, Panama Tall; LYHT, Hawaiian Tall; LYMAY, Maypan hybrid (Malayan Dwarf × Panama Tall). HMD, and HJT, healthy Malayan Dwarf and Jamaica Tall, respectively. (B) LY-affected palm species: LYVM (1-5), five *Veitchia merrillii* (Manila) palms; LYPD (1-3), three *Phoenix dactylifera* (true date) palms; LYPS, *P. sylvestris* (silver date); LYTF, *Trachycarpus fortunei* (windmill palm); LYLC, *Livistona chinensis* (Chinese fan palm); LYLR, *L. rotundifolia* (footstool palm); LYCR, *Caryota rumphiana* (giant fishtail palm); LYHV, *Hyophorbe verschafeltii* (spindle palm); LYAE, *Arenga engleri* (dwarf sugar palm); LYDA *Dictyosperma album* (princess palm). HLC, HPD, HVM, healthy Chinese fan, true date and Manila palms, respectively. (C) Single inflorescences from LY-symptomatic coconut cultivars: LYJT(N), Jamaica Tall (Naples, FL); LYJT(M) Jamaica Tall (Miami, FL); LYMD (1-3), three Malayan Dwarf palms; LYMD(4A-G), seven successively younger inflorescences from a symptomatic Malayan Dwarf; LYLC, inflorescence from *L. chinensis*. Immature inflorescences from symptomless (apparently healthy) *C. nucifera* cultivars: HMD, Malayan Dwarf; HIGD, Indian Green Dwarf cultivars; WC, water control; E H, lambda DNA *Eco*RI–*Hin*dIII fragments; 123, BRL 123 bp ladder.

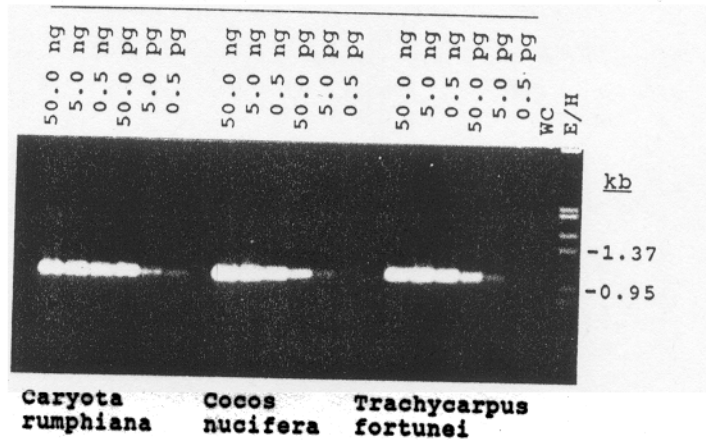

Fig. 6. Detection of the lethal yellowing (LY) mycoplasma-like organism DNA by PCR using pathogen-specific primers and PCR template DNA derived from heart tissues of three lethal yellowing-affected palm species. Following 35 cycles of amplification, one-fifth of each reaction mixture was analysed by 10 mg/ml agarose gel electrophoresis. WC, water control; E H, lambda DNA *Eco*RI–*Hin*dIII fragments.

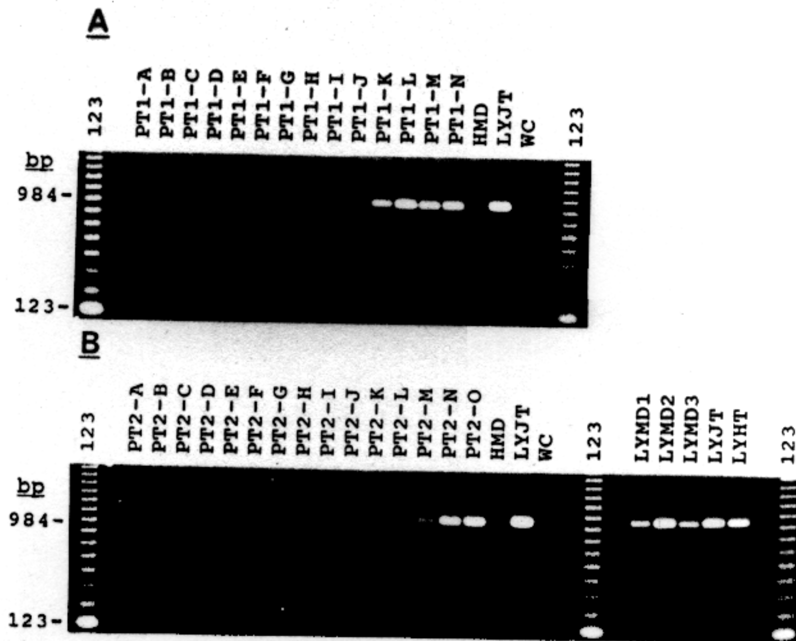

Fig. 7. Detection of lethal yellowing mycoplasma-like organism DNA by PCR in leaves of *Cocos nucifera* (coconut) cultivars with primary or yellowing stage symptoms. Template DNA for PCR was derived from laminae of basal leaflets from successively younger leaves of two Panama Tall (PT) cultivars with early foliar yellowing (A,B) and from the youngest (spear) leaf only of five additional palms (B). PT1, PT2, Panama Tall (A–N or A–O, oldest to youngest leaf, respectively); LYMD1, Malayan Dwarf with primary phase symptoms; LYMD(2-3), Malayan Dwarfs with early yellowing phase symptoms; LYJT, Jamaica Tall with spear necrosis only; LYHT, Hawaiian Tall with early yellowing. HMD, healthy Malayan Dwarf; LYJT, heart tissue of lethal yellowing-diseased Jamaica Tall; WC, water control; 123, BRL 123 bp ladder.

DISCUSSION

The goal of this work was to develop primers for use in LY MLO-specific PCR. The exceptional sensitivity of PCR offers many advantages for detection of plant pathogens (Henson & French, 1993). Application of this technique to detection and investigation of the lethal yellowing MLO seems particularly appropriate because of the very low titres of the MLO in mature palm tissues. Previous studies of lethal yellowing disease have been complicated by several other factors including an inability to culture the pathogen *in vitro*; the ability of the disease to kill palms quickly, and an absence of a means to perpetuate efficiently the disease. Palms remain as the sole source of the lethal yellowing MLO for study since no alternative plant hosts have been identified. Similarily, both presence and multiplication of the lethal yellowing MLO in its putative insect vector, *M. crudus*, and experimental transmission of the MLO to other plants have yet to be demonstrated.

In order to achieve the desired specificity to the PCR we first cloned and identified genomic DNA unique to the lethal yellowing MLO from which sequence information could be exploited for primer design. Detection of MLOs in plant tissues by PCR using primers based upon MLO 16S rRNA sequences has been reported (Deng & Hiruki, 1991b; Ahrens & Seemüller, 1992; Schneider *et al.*, 1993). However, none of these primer sets has thus far enabled pathogen-specific detection, thereby limiting their possible use in searches using PCR for insect and alternative plant hosts of MLOs.

Evaluation of cloned fragments of lethal yellowing MLO DNA by dot hybridizations, using enriched DNA of the western X-MLO and pigeon pea witches' broom MLO as probes, and then by using cloned MLO DNA fragments as probes in reciprocal hybridizations, proved to be a suitable method for identifying lethal yellowing MLO-specific DNA sequences. That we were successful with this approach was attributed to choice of these two particular MLOs for use as both probe and target DNAs. Their selection was based upon recent comparisons of MLO ribosomal RNA gene sequences which indicated close relationships between both MLOs and the lethal yellowing MLO (Ahrens *et al.* 1992; N. A. Harrison, unpublished data).

For the design of pathogen-specific primers, the foremost consideration was to use genomic DNA sequences which would permit amplification of a lethal yellowing MLO DNA product of sufficient size (*c.* 1 kbp) that could be conveniently resolved by electrophoresis in standard agarose gels. This was effectively accomplished by selecting a primer pair from sequences of an appropriately sized subclone derived from a larger lethal yellowing MLO-specific probe. The specificity of the chosen primer set was verified during initial PCR in which they failed to amplify any product from target DNAs extracted from other MLOs indigenous to Florida and California, culturable mollicute contaminants of palms, phytopathogenic spiroplasmas, and MLOs associated with African coconut lethal decline diseases. Lack of detection of the last of these Old World coconut pathogens by our PCR complements recent studies which revealed these MLOs to be similar but not identical to the lethal yellowing MLO (Ahrens *et al.*, 1992, Harrison *et al.*, 1994).

Detection of the lethal yellowing MLO was consistent and reproducible for all affected palm species and cultivars examined when template DNAs for PCR were derived from either unemerged inflorescences or hearts. These results agree with earlier ultrastructural observations of MLO distribution in lethal yellowing-diseased palms (Waters & Osborne, 1978; Thomas & Norris, 1980). However, inflorescences are not always available for sampling as immature palms frequently contract lethal yellowing (McCoy *et al.*, 1983). Also, removal of hearts and concomitant death of palms is a particularly unsuitable sampling practice in questionable cases of disease for which diagnostic information provided by PCR has potentially the greatest value. Spear leaves and roots have been reported to contain MLOs once distal portions of these organs showed evidence of necrosis (Waters & Osborne, 1978). The pathogen was rarely observed in mature foliage (Thomas & Norris, 1980) with the exception of flag leaves, in which MLO distribution appeared to be restricted to yellowed portions only (Waters & Osborne, 1978).

In the present study, detection of lethal yellowing MLO infections in predominantly non-symptomatic coconut leaves by PCR confirmed the effectiveness of this technique and indicated that sampling of foliage could provide the necessary practical means for diagnosing lethal yellowing disease in this palm species. The capacity of PCR to detect the lethal yellowing MLO was greatly enhanced by using leaflets of the least mature leaves. Our detection success, in

limited sampling, was 100% for leaflets removed at random from the mid to lower portions of the spear, the most immature leaf. We purposely chose palms exhibiting the earliest visible symptoms indicative of lethal yellowing in order to challenge the detection sensitivity of PCR. Thus, for palms such as the Panama Tall cultivar with early yellowing phase symptoms, necrosis and yellowing were present only in distal portions of one or two of the most mature leaves, leaving basal leaflets unaffected. We were unable to detect the pathogen in these leaflets. However, during a recent investigation of MLO acquisition by phloem-feeding homopterans from coconut palms with mid- to late-stage yellowing symptoms, we frequently detected MLO in yellowed leaflets from mature leaves by PCR (N. A. Harrison, unpublished data). Therefore, our inability to locate the pathogen in mature leaves suggests perhaps an uneven distribution of MLO rather than its absence from these organs.

The sensitivity and convenience of the lethal yellowing MLO-specific PCR should encourage further research on lethal yellowing epidemiology such as detailed studies of vector biology, host resistance and identification of alternative plant hosts which have previously been beyond the reach of the available technology.

ACKNOWLEDGEMENTS

We are grateful to T. K. Broschat and F. W. Howard, University of Florida, for providing us with samples of healthy and diseased palms. We also thank J. A. Wyman, B. C. Kirkpatrick, M. A. Villanueva and P. Jones for providing additional MLO-infected plants or MLO DNAs and R. F. Whitcomb and J. G. Tully for providing culturable mollicutes. Support for this research was provided by USDA Tropical/Subtropical Agriculture Special Grants Program (grant No. 90-34135-5165 and 92-34135-7281). This paper is published as Florida Agricultural Experiment Station journal series No. R-03392.

REFERENCES

Ahrens U, Seemüller E, 1992. Detection of DNA of plant pathogenic mycoplasmalike organisms by a polymerase chain reaction that amplifies a sequence of the 16S rRNA gene. *Phytopathology* **82**, 828–32.

Ahrens U, Seemüller E, Kirkpatrick BC, Gao J-L, Harrison N, 1992. Detection and differentiation of plant pathogenic MLOs using PCR and MLO-specific 16S rRNA primers. *IOM Letters* **2**, 25.

Anonymous, 1986. Palm lethal yellowing mycoplasm. *EPPO Bulletin* **16**, 61–5.

Davis MJ, Tsai JH, Cox RL, McDaniel LL, Harrison NA, 1988. Cloning of chromosomal and extrachromosomal DNA of the mycoplasmalike organism that causes maize bushy stunt disease. *Molecular Plant–Microbe Interactions* **1**, 295–302.

Davis MJ, Tsai JH, McCoy RE, 1984. Isolation of the corn stunt spiroplasma from maize in Florida. *Plant Disease* **68**, 600–4.

Dellaporta SL, Wood J, Hicks JB, 1983. A plant DNA minipreparation: Version II. *Plant Molecular Biology Reporter* **1**, 19–21.

Deng S, Hiruki C, 1991a. Genetic relatedness between two nonculturable mycoplasmalike organisms revealed by nucleic acid hybridization and polymerase chain reaction. *Phytopathology* **81**, 1475–9.

Deng S, Hiruki C, 1991b. Amplification of 16S rRNA genes from culturable and nonculturable mollicutes. *Journal of Microbiological Methods* **14**, 53–61.

Doyle JJ, Doyle JL, 1990. Isolation of plant DNA from fresh tissue. *Focus* **12**, 13–15.

Eden-Green SJ, Tully JG, 1979. Isolation of *Acholeplasma* spp. from coconut palms affected by lethal yellowing disease in Jamaica. *Current Microbiology* **2**, 311–16.

Eden-Green SJ, Waters H, 1981. Isolation and preliminary characterization of a spiroplasma from coconut palms in Jamaica *Journal of General Microbiology* **124**, 263–70.

Harrison NA, Tsai JH, Bourne CM, Richardson PA, 1991. Molecular cloning and detection of chromosomal and extrachromosomal DNA of the mycoplasmalike organisms associated with witches' broom disease of pigeon pea in Florida. *Molecular Plant–Microbe Interactions* **4**, 300–7.

Harrison NA, Bourne CM, Cox RL, Tsai JH, Richardson PA, 1992. DNA probes for detection of mycoplasmalike organisms associated with lethal yellowing disease of palms in Florida. *Phytopathology* **82**, 216–24.

Harrison NA, Richardson PA, Jones P, Tymon AM, Eden-Green SJ, Mpunami AA, 1994. Comparative investigation of mycoplasmalike organisms associated with Caribbean and African coconut lethal decline diseases by DNA hybridizations and PCR assays. *Plant Disease* **78**, 507–11.

Henson JM, French R, 1993. The polymerase chain reaction and plant disease diagnosis. *Annual Review of Phytopathology* **31**, 81–109.

Hibben CR, Sinclair WA, Davis RE, Alexander JH, 1991. Relatedness of mycoplasmalike organsims associated with ash yellows and lilac witches' broom. *Plant Disease* **75**, 1227–30.

Howard FW, 1983. World distribution and possible geographic origin of palm lethal yellowing and its vectors. *FAO Plant Protection Bulletin* **31**, 101–13.

Howard FW, Norris RC, Thomas DL, 1983. Evidence of transmission of palm lethal yellowing agent by a planthopper *Myndus crudus* (Homo-

ptera: Cixiidae). *Tropical Agriculture (Trinidad)* **60**, 168–71.

Kollar A, Seemüller E, Bonnet F, Saillard C, Bové JM, 1990. Isolation of the DNA of various plant pathogenic mycoplasmalike organisms from infected plants. *Phytopathology* **80**, 233–7.

Lee I-M, Davis RE, 1988. Detection and investigation of genetic relatedness among aster yellows and other mycoplasmalike organisms using cloned DNA and RNA probes. *Molecular Plant–Microbe Interactions* **1**, 303–10.

Lee I-M, Davis RE, Sinclair WA, DeWitt ND, Conti M, 1993a. Genetic relatedness of mycoplasmalike organisms detected in *Ulmus* spp. in the United States and Italy by means of DNA probes and polymerase chain reactions. *Phytopathology* **83**, 829–33.

Lee I-M, Hammond RW, Davis RE, Gunderson DE, 1993b. Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasmalike organisms. *Phytopathology* **83**, 834–42.

Liao CH, Chen TA, 1977. Culture of corn stunt spiroplasma in a simple medium. *Phytopathology* **67**, 802–7.

McCoy R, Davis MJ, Dowell RV, 1981. *In vivo* cultivation of spiroplasmas in larvae of the greater wax moth. *Phytopathology* **71**, 408–11.

McCoy RE, Howard FW, Tsai JH, Donselman HM, Thomas DL, Basham HG, Atilano RA, Eskafi FM, Britt L, Collins ME, 1983. *Lethal Yellowing of Palms*. Gainesville, FL, USA: University of Florida, IFAS, Agricultural Experiment Station Bulletin No. 834.

McCoy RE, Thomas DL, 1980. Periwinkle witches' broom disease in south Florida. *Proceedings of the Florida State Horticultural Society* **93**, 179–81.

Namba S, Oyaizu H, Kato S, Iwanami H, Tsuchizaki T, 1993. Phylogenetic diversity of phytopathogenic mycoplasmalike organisms. *International Journal of Systematic Bacteriology* **43**, 461–7.

Sambrook J, Fritsch EF, Maniaitis, T, 1989. *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory Press.

Schaff D, Lee I-M, Davis RE, 1992. Sensitive detection and identification of mycoplasmalike organisms in plants by polymerase chain reaction. *Biochemical and Biophysical Research Communications* **186**, 1503–9.

Schneider B, Ahrens U, Kirkpatrick BC, Seemüller E, 1993. Classification of plant pathogenic mycoplasma-like organisms using restriction-site analysis of PCR-amplified 16S rDNA. *Journal of General Microbiology* **139**, 519–27.

Sinclair WA, Griffiths HM, Davis RE, Lee IM, 1992. Detection of the ash yellows mycoplasmal organisms in different tree organs and in chemically preserved specimens by a DNA probe vs DAPI. *Plant Disease* **76**, 154–8.

Thomas DL, 1979. Mycoplasmalike bodies associated with lethal declines of palms in Florida. *Phytopathology* **69**, 928–34.

Thomas DL, Norris RC, 1980. The use of electron microscopy for lethal yellowing diagnosis. *Proceedings of the Florida State Horticultural Society* **93**, 196–9.

Tully JG, Whitcomb RF, Clark AF, Williamson DF, 1977. Pathogenic mycoplasmas: cultivation and vertebrate pathogenicity of a new spiroplasma. *Science* **195**, 892–4.

Vega FE, Davis RE, Barbosa P, Dally EL, Purcell AH, Lee I-M, 1993. Detection of a plant pathogen in a nonvector insect species by the polymerase chain reaction. *Phytopathology* **83**, 621–4.

Waters H, Osborne I, 1978. Preliminary studies upon lethal yellowing and the distribution of MLO in coconut palms. In: *Proceedings of the 3rd Meeting of the International Council on Lethal Yellowing*. Gainesville FL, USA: University of Florida, IFAS, Agricultural Experiment Station, Publication FL-78-2, p. 13.

Annals of Botany **79**: 31–40, 1997

A Humidity-induced Convective Throughflow Ventilation System Benefits *Annona squamosa* L. Explants and Coconut Calloid

J. ARMSTRONG*, E. E. P. LEMOS†, S. M. A. ZOBAYED*, S. H. F. W. JUSTIN† and W. ARMSTRONG*

* *Department of Applied Biology, University of Hull, Hull, HU6 7RX and* † *Unit for Advanced Propagation Systems, Wye College, University of London, Nr. Ashford, Kent, TN25 5AH, UK*

Received: 23 April 1996 Accepted: 23 July 1996

A simple apparatus is described for generating pressurized throughflow ventilation in plant tissue culture vessels. No pumps or gas-cylinders are required and the flow is driven by humidity-induced diffusion across microporous membranes. In the experiments described, pressurized flows of sterile humidified air were supplied at rates of up to 1 ml min^{-1} and these had beneficial effects on leaf survival and production in *Annona* cuttings and on calloid form in coconut. Ethylene (ethene) was removed more quickly from the pressure-flow ventilated culture vessels (t_{50}, 0·4–0·7 h) than from those aerated by diffusion through conventional polypropylene membranes (t_{50}, 1·6–2·4 h).

In *Annona* cuttings leaf production was greatly increased and ethylene-induced leaf fall considerably delayed when cultured with the forced as opposed to diffusion-based ventilation of the vessels.

With throughflow ventilation, coconut calloid was more convoluted than under wholly diffusive aeration and had a smooth distinct epidermal surface and clearly defined sub-epidermal meristematic nodules. It resembled freshly initiated calloid from which regeneration of plantlets via somatic embryogenesis can be obtained. Under wholly diffusive aeration, calloid developed a rough, relatively undifferentiated surface, more haustorial (i.e. cotyledonary) in appearance, and characteristic of cultures where regeneration potential has been lost.

It is suggested that other benefits of the pressurized throughflow ventilation may be the removal of volatiles such as ethanol and acetaldehyde, the removal of excess carbon dioxide at night and its improved supply during the day, and sustained oxygen concentrations at levels close to atmospheric both night and day.

© 1997 Annals of Botany Company

Key words: Abscission, callus, ethylene, explants, tissue-culture, growth, ventilation.

INTRODUCTION

Widespread concern has been expressed recently regarding the adequacy of the ventilation achieved in some conventional plant tissue-culture systems (Woltering, 1986; Jackson *et al.*, 1987; Adkins, 1992; Debergh *et al.*, 1992; Righetti and Facini, 1992; Rossetto, Dixon and Bunn, 1992). Current methods for ventilating callus, explants and cuttings in vessels under sterile conditions often rely upon the diffusion of gases through microporous membranes, or gas-permeable films, at the mouth or in the wall of the vessel. Alternatively, in sealed vessels, aeration may be dependent upon photosynthetically generated oxygen. Culturing in diffusive ventilation or in sealed vessels, although preventing the contamination of the culture by air-borne bacteria or fungal spores, can lead to the accumulation of acetaldehyde, ethylene (ethene) and ethanol (Thomas, Des and Murashige, 1979; Righetti, Magnanini and Maccaferri, 1987; Righetti, Magnanini and Infante, 1990), while concentrations of carbon dioxide and oxygen may be lowered or raised from their physiological optima (De Proft, Maene and Debergh, 1985). Some species are especially susceptible to ethylene accumulation. For example, Jackson *et al.* (1991) found that *Ficus lyrata* and *Solanum tuberosum* cv. Red Craig's Royal (potato) were adversely affected by poor aeration in tightly sealed culture vessels. After 28 d, the leaf area of *Ficus* was reduced by more than 50%, while shoot height in *Solanum* was reduced by 64% after 14 d, and leaf expansion was minimal. Jackson *et al.* (1991) concluded that accumulated ethylene was responsible for these effects, since in both species the use of an ethylene absorbent or antagonist in the tightly sealed vessels led to normal growth, indistinguishable from that in loosely sealed vessels where the endogenously generated ethylene could escape easily.

The benefits of enhanced diffusive ventilation for *Malus domestica* seedlings as well as potato explants were emphasized in a subsequent paper (Jackson, Belcher and Brain, 1994). Also, Rossetto *et al.* (1992) found that improved diffusive aeration of tissue-cultured rare Australian plants, including species of *Conostylus*, *Diplolena*, *Drummondita*, *Lechenaultia*, and *Sowerbaea*, reduced vitrification and significantly improved shoot quality and the later establishment of plants in soil. Buddendorf-Joosten and Woltering (1996) have reported on an improved diffusion-based ventilating system for potato plantlets in which the atmosphere surrounding the culture vessels was constantly refreshed and humidity-controlled.

An alternative to diffusive ventilation is to use forced ventilation of the culture vessels, and it is becoming increasingly apparent that forced ventilation can have a number of advantages over conventionally sealed systems or those relying on gaseous diffusion (Kozai, Kitaya and

0305-7364/97/010031 + 10 $25.00/0 bo960299 © 1997 Annals of Botany Company

Kubota, 1995): growth of cuttings, seedlings and callus can all be improved. For example, Kozai, Kubota and Nakayama (1989) found higher photosynthetic rates and growth of strawberrry plants *in vitro* with forced ventilation than with conventional *in vitro* conditions, as did Yue, Gosselin and Desjardins (1993) using *Pelargonium* cuttings. Adkins (1992) found that rice callus benefitted from a forced ventilation of the head-space of the tissue culture vessels and attributed the improved performance to ethylene removal and an improved oxygen regime. However, the systems used to achieve forced ventilation are usually rather complex and require compressed gases or electrically-driven pumps, filtration systems, and often gas-mixing/metering devices to maintain the flow balance to the culture vessels.

In this paper we describe an alternative non-mechanized apparatus for achieving forced ventilation. The apparatus delivers a sustained stream of sterile humidified air driven by the process of humidity-induced diffusion. This process, which depends only upon the maintenance of a water vapour gradient across a microporous membrane, is also a major cause of pressurized gas-flows in a number of wetland and aquatic macrophytes (Dacey, 1981; Armstrong and Armstrong, 1990; Armstrong *et al.*, 1990; Brix, Sorrell and Orr, 1992).

We report on the effects of the pressurized ventilation on the growth of explants of the ethylene-sensitive species *Annona squamosa* L. (Lemos and Blake, 1994), and on the growth and differentiation of the recalcitrant calloid of coconut. In the case of *Annona*, the culture medium was prepared either with or without an ethylene antagonist. The system is compared with conventional diffusive ventilation of tissue cultures using polypropylene membranes; also, the rates of ethylene efflux from, and its accumulation within, culture vessels ventilated in various ways are reported.

THE PRESSURE-FLOW VENTILATING APPARATUS AND THE UNDERLYING PRINCIPLES INVOLVED

Humidity-induced pressure flow (convective throughflow) in the wetland plant *Phragmites australis* (Cav.) Trin. ex Steud., is driven by a diffusion of oxygen and nitrogen through the stomata of the leaf sheaths into the humid atmosphere of the substomatal cavities (Armstrong and Armstrong, 1990). Here, the constant humidification of the internal atmosphere (up to 2–3% by volume) creates and maintains water vapour levels, thus diluting the atmospheric gases oxygen and nitrogen and producing a concentration gradient for their inward diffusion from the drier outer air. If stomatal resistance to inward diffusion is effectively less than any Poiseuille resistance to backflow to the atmosphere, the inwardly diffusing air will cause a pressurization within the plant. If there was no other path for gases to escape, the total pressure within the leaf sheath gas space at equilibrium would be greater than atmospheric by an amount numerically equal to the water vapour partial pressure beneath the stomata. In plants such as *Phragmites*, however, there is an alternative outflow path, and the tendency to pressurize drives a convective flow (pressure-flow) of gases into the underground parts and back to the atmosphere through old

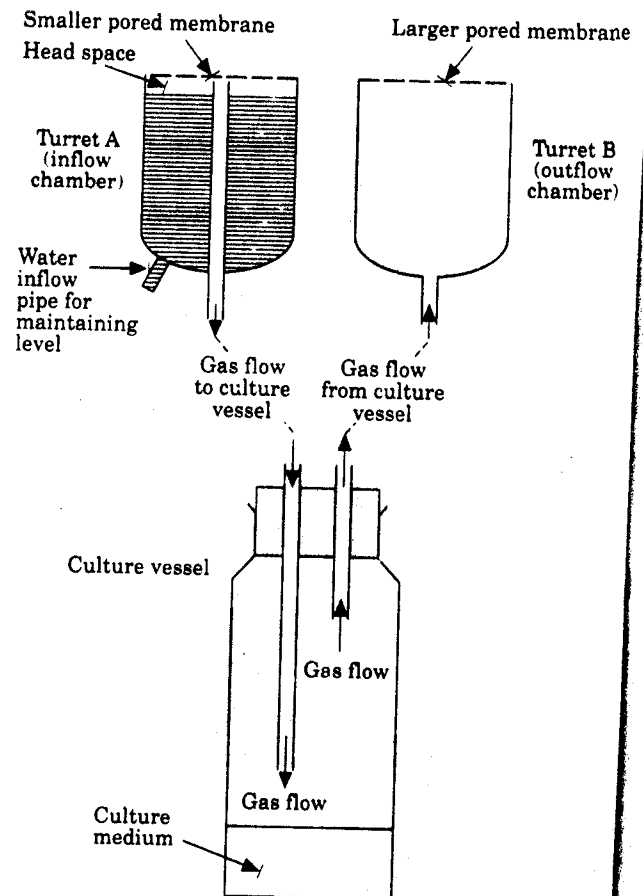

FIG. 1. Pressurized ventilating apparatus for continuous supply of humidified sterile air to the head-space of tissue culture vessels; the pressure-flow mechanism depends upon humidity-induced diffusion of atmospheric air into turret A through small-pored Nuclepore membrane (pore diameters 0·03 μm). Venting is through the larger-pored membrane (pore diameters 0·2 μm) on turret B. A water-filled syringe permanently attached to the water-inflow pipe may be used for maintaining the water level in Turret A. In this example the gas-flow into and from the culture vessel is via glass tubes.

dead flowering shoots—hence the term convective throughflow.

It was envisaged that a simple ventilation system based on the principle of the humidity-induced convective throughflow found in *Phragmites* could be devised to provide a sterile flow of air to tissue culture vessels if two chambers, each bearing a microporous membrane, were combined. A smaller-pored, humidified inflow chamber would induce the flow, while venting would take place via a dry outlet chamber. In the humidity-induced pressure-flow ventilating apparatus described here (Fig. 1), the pressurization and sterile humidified gas-flow was created using microporous Nuclepore membranes (pore diameters 0.03 μm; thickness 10 μm; membrane porosity approx. 10%) overlying, but not touching, a reservoir of water in a glass 'inflow turret' (turret A: internal diameter, 20 mm; length, 30 mm). A glass 'outflow tube' opening into the humidified head-space of turret A, just below the membrane, directs the incoming gases to the header space of the culture vessel. Venting to the atmosphere occurs through a second, 'dry' turret (outlet

turret B) capped by a larger pored Nuclepore or Millipore membrane (pore diameter. 0·2 μm; porosity and thickness as for turret A). This pore size allows the gases to be vented easily under the pressure differentials generated by the inflow turret, and maintains the sterility of the apparatus. A syringe containing sterile water for filling the turret and adjusting the water level is connected to a side-arm on turret A.

A patent for this type of apparatus (No. GB227505B–UK Patents Office) has recently been granted (Armstrong and Armstrong, 1996). Details of the functioning of Nuclepore membranes for inducing pressurizations and gas-flows are to be found in Armstrong (1992), Armstrong and Armstrong (1994) and Armstrong, Armstrong and Beckett (1996*a*, *b*).

MATERIALS AND METHODS

Plants were grown in glass culture tubes, (length 75 mm, i.d. 23 mm, capacity 30 ml), fitted with either, (*a*) a conventional diffusive ventilator consisting of a disc of polypropylene film (thickness 25 μm: oxygen transmission rate, $< 1{\cdot}8 \times 10^{-2}$ m^3 m^{-2} d^{-1} MPa^{-1}: Courtaulds Films, Bridgewater, Somerset, UK), secured over the mouth of the tube by a rubber band, or (*b*) the humidity-induced pressure-flow ventilating system (Fig. 1). The reservoir water level in the pressure-flow assembly was raised initially to within 1–2 mm of the membrane each day, but since the water level tended to fall by 1–4 mm during 24 h, it was re-adjusted to the original level at the beginning of each day. For throughflow ventilation, the culture tube was fitted either with a 'Suba-seal' cap penetrated by two hypodermic needles to serve as outflow and inflow paths to and from the turrets, or with a rubber bung and two glass tubes, the longer tube being for the inflow. Each of these tubes was connected to a turret by silicone rubber tubing of wall thickness 1·4 mm (Fig. 1).

Under the conditions of most of the experiments: 25–29 °C and 40–60% relative humidity (RH), the ventilation rate for a throughflow system, starting with the water close to the membrane (1–2 mm below) at the beginning of the day, ranged between 0·5 and 1·0 ml min^{-1}. Thus, at the fastest flow rate, the air could be 'renewed' approximately every 30 min in a 30 ml culture tube.

Throughflow ventilation rates

Various procedures were used to characterize the functioning of the assembled pressurized ventilating system. Firstly, gas flows within the assembly were measured using a soap film flow meter joined in series with the down-pipe from the inflow turret to the culture vessel. Flow measurements were made with culture vessels (*a*) empty, (*b*) containing 5 ml agar, (*c*) containing 5 ml agar together with a leafy cutting, and (*d*) with the culture vessel empty and the inflow turret A dry. Growth room conditions were 25 °C and 17% RH, and there were four replicates per treatment.

Secondly, flows were measured with and without an outflow turret and with the distance between the water surface and inflow membrane varied between 2 and 19 mm. Growth room conditions were 21·5 °C and 36% RH, and there were four replicates per treatment. Here the culture vessels were empty.

Ventilating efficiencies—t_{50} measurements using ethylene

The efficiency of ventilating systems can be compared by measuring the time taken, (t_{50}), for half of an injected standard sample of a marker gas, e.g. ethylene, to escape from the vessel (Jackson *et al.*, 1991). The t_{50}s were measured with the culture vessels capped in various ways, using: (*a*) 'Suba-seal' rubber puncture caps, (*b*) cling-film PVC (thickness, 10 μm), (*c*) conventional polypropylene discs (thickness, 25 μm), (*d*) the convective ventilation apparatus with hypodermic needles, (*e*) the convective ventilation apparatus with glass tubing, and (*f*) as for (*e*), but with inflow turret A dry. Two sets of comparisons were made, the first (set A) involved systems a–e, the second (set B) involved only c, e and f.

For set A, the vessels were open ended tubes, similar in size to the culture tubes, and were 'capped' at the top as described above and at the bottom with a 'Suba-seal' rubber puncture cap; gas samples were added or withdrawn through this using a hypodermic syringe. At each addition or removal of gas, an equal volume of chamber-gas or air was respectively removed or added to maintain a constant pressure in the assembly. Five of each type of assembly were used as replicates, and 0·21 ml volumes of 1% ethylene in nitrogen were injected through the lower 'Suba-seals' sufficient to create an initital concentration of ethylene in each assembly of 70 μl l^{-1}. Ethylene concentrations were then determined at regular intervals by removing 0·5 ml samples of gas from the assemblies and analysing by means of gas chromatography (Vega 6000-GC, Carlo Erba Strumentazione). Porapack (80–100 mesh) was used in a stainless steel column (800 × 1·5 mm), and column, injector and flame ionization detector temperatures were 80, 150 and 150 °C, respectively. Nitrogen was used as the carrier gas at a rate of 50 ml min^{-1}. The time taken (t_{50}) for the escape of 50% of the ethylene was determined from plots of ethylene concentration against time. Growth room conditions were 25 °C and 40–60% RH, and the inflow membrane pore diameters were 0·03 μm.

For set B normal culture vessels were used, but each had a side arm with a 'Suba-seal' for adding or removing gas samples. Vessels were initially filled with 23 μl l^{-1} ethylene and 500 μl samples were removed for analysis at various time intervals depending upon the ventilation system being tested. However, each vessel was sampled only once to avoid artificially diluting the sample. To this end nine vessels per treatment were used for each run, and the runs were replicated four times. The ethylene samples were analysed by GC (Pye Unicam, Model GCD) using Poropack Q (60–80 mesh) in a glass column (2500 × 6·5 mm) at 100, 150 and 150 °C. The peak (which was removable by pre-exposure of samples to permanganate) was identified by a retention time of about 1·4 min and nitrogen was used as carrier gas at a rate of 60 ml min^{-1}. The ventilating assemblies were in a growth room at 27 °C and 25% RH.

Growth of Annona *explants with diffusive or convective ventilation and with or without an ethylene antagonist*

Annona nodal cuttings of 20 mm, from new fluxes of mature plants previously grown in a glass house, were transferred, one to each culture tube containing 8 ml of MS (Murashige and Skoog, 1962) medium with 20 mg l^{-1} sucrose, 2 mg l^{-1} BAP, 3 g l^{-1} Phytagel and pH corrected to 5·8 prior to autoclaving.

Replicates (four per treatment), were grown for 6 weeks in a growth room with: (*a*) diffusive ventilation without an ethylene antagonist, (*b*) diffusive ventilation with the ethylene antagonist, silver thiosulphate ($Ag_2S_2O_3$, 0.5 mg l^{-1} of culture medium, i.e. 3·06 μmol Ag l^{-1}), (*c*) convective flow ventilation without an ethylene antagonist, and (*d*) convective flow ventilation with silver thiosulphate (0·5 mg l^{-1} of culture medium). Growth room temperature was 25 ± 1 °C, PAR approx. 100 μmol m^{-2} s^{-1}, day-length 12 h and relative humidity 40–60%.

The numbers of leaves produced, and leaf abscission, expressed as a percentage of leaves produced, were recorded for each week of the experiment.

The choice of $Ag_2S_2O_3$ concentration was made after testing plants over a range of concentrations (0·01, 0·05, 0·1, 0·5, 1·0, 2·0, and 5·0 mg l^{-1}). The optimum concentration was 0·5 mg l^{-1}; below this there was some leaf abscission, while above it there was an increasing inhibition of new leaf production and growth.

Effects of various methods of ventilation on ethylene accumulation in culture vessels containing Annona *cuttings*

Culture vessels (30 ml), each containing 5 ml of nutrient medium as above, and an *Annona* cutting (f.wt. 357 ± 56 mg; leaf area 10·8 ± 2·6 cm^2) were capped with (*a*) a silicone rubber bung, (*b*) conventional polypropylene film or (*c*) the throughflow ventilation apparatus. Each vessel had a side-arm and Suba-seal for removing gas samples as described previously. Five replicates were prepared per treatment and kept for 48 h at 23 °C, 80 μmol m^{-2} s^{-1} PAR and 37% RH. Gas samples (500 μl) were removed at intervals and analysed for ethylene concentrations as described for set B.

The production of recalcitrant long-term coconut calloid and application of diffusive or pressure-flow ventilation

When cultured on modified medium (Murashige and Skoog, 1962) containing 0·25% activated charcoal and 1×10^{-4} M 2,4-D, embryogenic coconut calloid derived from rachillae of the cv. Malayan Dwarf may, on reduction of auxin levels, form somatic embryos and plantlets (Branton and Blake 1983*b*, 1986). However, abnormal development can occur; one of the most common forms being the predominant development of spongy growth resembling the haustorium (or cotyledon) which normally enlarges inside the nut supplying the developing zygotic embryo (Fremond, Ziller and de Nuce de Lamothe, 1966; Branton and Blake 1983*a*). Cultures developing haustorial growth may become embryogenic once again by increasing the 2,4-D level to 4×10^{-4} M for 1 or 2 months. Plantlets have been produced from such recycled material (Blake and Wilson, 1990). Such embryogenic calloid is smooth and shiny (Branton and Blake, 1986). Often, however, a form of long-term calloid develops which continues to divide. This can be subcultured frequently, but is no longer strictly embryogenic, i.e. although a type of calloid continues to form, it can be rougher than true calloid and no longer produces somatic embryoids spontaneously. The use of the pressure-flow ventilating system was tested on such long term coconut calloid in an attempt to return it to embryogenic calloid with embryo and plantlet formation.

Coconut calloid from cv. Malayan Dwarf (Blake and Hornung, 1994) was first cultured on modified Murashige and Skoog medium (Brackpool, Branton, and Blake, 1986) but with BAP omitted and the addition of 2iP at 1×10^{-6} M, 2,4-D at 1×10^{-4} M, and 0·3% Gelrite used as gelling agent. Each calloid explant (volume approx. 1 ml) was then transferred into a culture tube containing 22 mm depth of modified MS medium, and subjected either to conventional diffusive or throughflow ventilation as described previously. Each treatment contained six replicates. Cultures were transferred or subcultured to fresh medium once a month and kept in the dark at 29 °C.

After 5 months, samples of the calloid from the two types of treatment were wax-embedded, sectioned, stained with haematoxylin and eosin, and examined by light microscopy. Photomicrographs were obtained using an Olympus IMT-2 inverted microscope with camera attachment.

For wax embedding, the specimens were dehydrated using an ethanol series and 'Histo-clear' prior to embedding in Fibrowax (Raymond A. Lamb formulation from MERCK Ltd., Poole, England BH15 1TD).

RESULTS AND DISCUSSION

Rates of throughflow ventilation

As indicated earlier, ventilation rates are at a maximum when the water surface is very close to the membrane of the inflow turret and decrease with time as the water level falls due to evaporation (Fig. 2). This figure also shows that the resistance imposed by the presence of the outflow membrane does not greatly reduce flows.

The results in Table 1 document the throughflow ventilation rates in the assembly with the outflow membrane in position and show that flow rates are relatively unaffected by the presence or absence of agar and a plant in the vessel. However, as expected, no throughflow occurs when the 'inflow turret' is dry, and in this case ventilation is by diffusion via both membranes; this latter case, relative to throughflow ventilation, is explored in the following section (set B).

Effects of different ventilation methods on the rates of ethylene escape from the culture vessel

The results presented in Table 2 indicate that convective throughflow ventilation is the most effective method for

removing ethylene from culture vessels, being more than twice as efficient as the best diffusive method normally used to cover the culture vessels, i.e. polypropylene discs. Cling film was a relatively poor membrane in terms of the diffusive loss of ethylene, while, as expected, the 'Suba-seal' was found to be highly impermeable: here the t_{50} values were more than 130 times larger than the convective flow system. By using hypodermic needles for inflow and outflow tubes, there is the danger of impeded gas flow from condensation droplets forming within them. In the case of the t_{50} experiments, however, the hypodermic needles had no significant effect on the t_{50} values obtained. In retrospect we were concerned also that the silicone rubber tubing used as

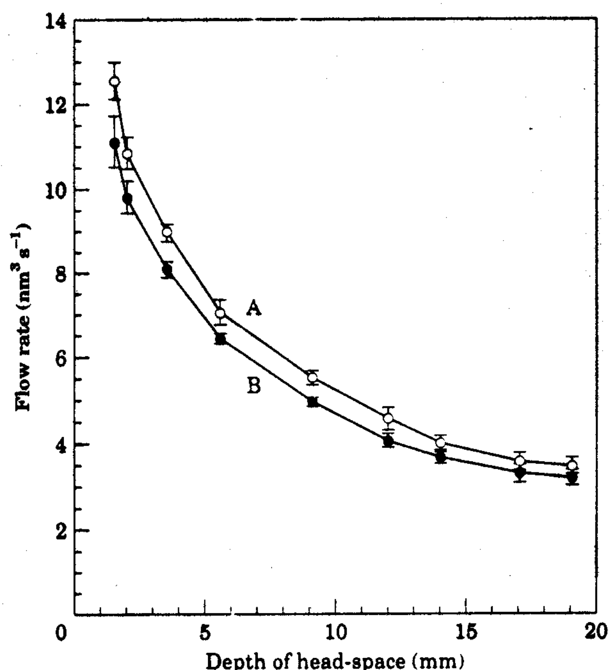

FIG. 2. Showing the effect of head-space depth (distance between membrane and underlying water surface) in the inflow turret on humidity-induced pressure flow ventilation rates: without outflow turret (○) and with outflow turret (●). Membrane pore diameters: inflow turret, 0·03 μm; outflow turret, 0·2 μm. Inside diameters of turrets, 20 mm; ambient temperature 21·5 °C; RH, 36%. Each point is the mean of four replicates; bars = ±s.e.

TABLE 1. *Ventilation rates through culture vessels. A, B, C: water surface < 2 mm below inflow membrane. Membrane pore diameters: inflow, 0·03 μm; outflow, 0·2 μm. Relative humidity, 17%; T, 25 °C. Data as means ± s.e.* (n = 3)

Culture Vessels	Inflow Turrent	Flow rates (nm³ s⁻¹)
A empty	humidified	17·03 ± 0·06
B + 5 ml culture medium	humidified	17·47 ± 0·22
C + 5 ml culture medium + plant	humidified	17·49 ± 0·22
D empty	dry	0

TABLE 2. *The influence of four methods of 'capping' plant tissue culture vessels on the time taken (t_{50}) for 50% of a sample of injected ethylene to be discharged from the system. Culture vessel volumes 30 ml; initial ethylene concentration approx. 70 μl l⁻¹. Relative humidity, 40–60%; T, 25 °C. Data as means ± s.e.* (n = 4)

Method of capping	t_{50} (h)
Suba-seal rubber puncture cap	5587 ± 95
Cling film (PVC)	1313 ± 40
Polypropylene discs + rubber band	94.8 ± 16
Convective flow apparatus (hypodermic needles as inflow and outflow tubes)	43 ± 29
Convective flow apparatus (glass inflow and outflow tubes)	30 ± 15

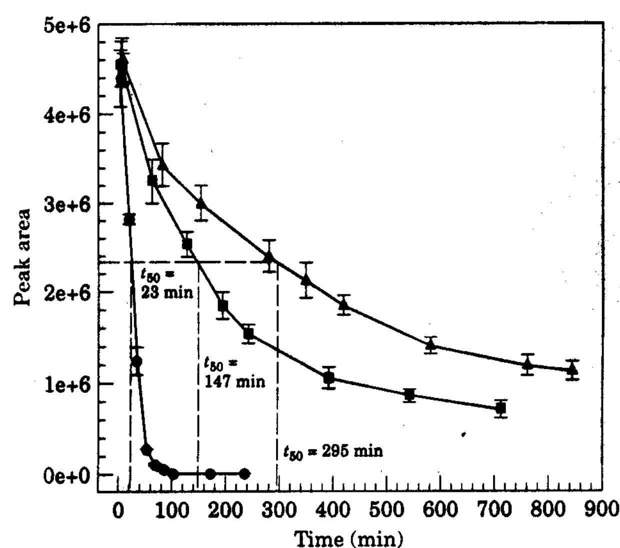

FIG. 3. The influence of various ventilating systems on the time taken (t_{50}) for ethylene at a concentration of 23 μl l⁻¹ in N_2 to be discharged from a 30 ml culture vessel: (●) throughflow ventilation apparatus (flow rate 1·0 ml min⁻¹); (■) culture vessel capped with polypropylene membrane; (▲) throughflow ventilation apparatus functioning only as a diffusive ventilating system (inflow and outflow turrets dry). Membrane pore diameters: inflow turret, 0·03 μm; outflow turret, 0·2 μm. Inside diameters of turrets, 20 mm; ambient temperature, 27 °C; RH, 25%. Each point is the mean of four replicates; bars = ±s.e.

part of the inflow and outflow paths in the convective flow assembly might have enhanced the rate of ethylene efflux from the culture vessels. Later studies in which less permeable PVC tubing was substituted for the silicone rubber showed that the influence of the silicone rubber on the t_{50} value was not statistically significant.

In Fig. 3, (data for set B), the t_{50} for the removal of ethylene by throughflow ventilation was only 23 min compared to 147 min for the polypropylene disc, and 295 min for the ventilation apparatus functioning diffusively. The reason for the latter functioning less effectively than the polypropylene was no doubt because the membranes were remote from the vessel and the ethylene had to negotiate the tubes and turrets before reaching the mem-

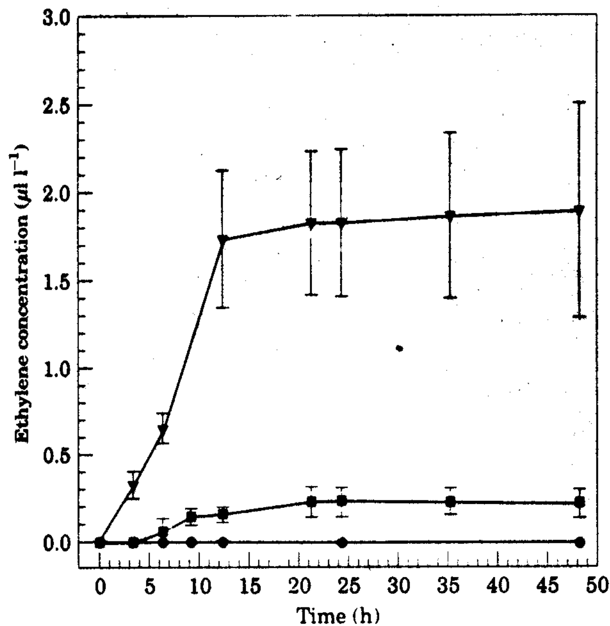

FIG. 6. Effect of culture vessel ventilating type on ethylene accumulation from *Annona squamosa* L. cuttings. Points show means of five replicates ± s.e. Leaf area of cuttings = 10·8 ± 2·6 cm^2; total fresh weight = 357 ± 56 mg; growth room temperature, 23 °C; RH, 37%; 30 ml culture vessels were used with 5 ml of medium. (▼) Sealed; (■) polyproplene membrane; (●) throughflow ventilation.

the plants grown in throughflow ventilation. This would accord with the effects of STS.

Ethylene accumulation in the polypropylene-capped vessels (Fig. 6) occurred only after 3 h and we believe may have been augmented by the development of condensation on the underside of the membrane. In our experience this is of common occurrence, and may be a major draw back of such systems. In wider 60 ml vessels the delay in ethylene accumulation was about 10 hours (data not shown) and probably accords with a slower development of condensation.

Effects of diffusive or convective ventilation on coconut calloid

After 20 weeks, the long-term calloid in the convective flow treatments produced a fine, more convoluted calloid, compared to the rougher, more haustorial, type of growth produced in diffusive ventilation. Histological sections showed that the calloid which had received convective ventilation was composed of smaller cells than the calloid subject to diffusive ventilation, and had a distinct epidermal layer, giving it a smooth, shiny appearance; in certain regions a distinct hypodermal epidermal layer was also present. This calloid also produced a profusion of clearly-defined sub-epidermal meristematic nodules, (Fig. 7A, C, E). In contrast, the calloid which had received only diffusive ventilation was composed of larger cells, the epidermis was poorly defined, and there was no obvious hypodermis; here, there were fewer and less discrete sub-epidermal meristematic nodules, (Fig. 7B, D, F). Also, in this callus, cells containing raphides were observed; these were absent from the calloid which had received throughflow ventilation. In both types of calloid, vascular elements with helical and scalariform thickenings were present.

FINAL DISCUSSION

Using t_{50}s as indicators, the throughflow method of ventilation at a relative humidity of 25% has been shown to be more than six times more efficient in facilitating the removal of ethylene from the culture vessels, compared to the most efficient conventional diffusive method investigated, i.e. via polypropylene discs (Fig.3). Even at a higher RH of 60%, the throughflow ventilation was > 2·4 times more efficient (Table 2). We have recent evidence (Zobayed, 1996) that this pressurized throughflow ventilation is similarly more efficient in sustaining CO_2 levels well above the compensation point during the day, and O_2 levels at night within the culture vessels. In terms of reducing ethylene accumulation, however, the throughflow ventilation can be regarded as very much more efficient than the polypropylene diffusive system (Fig. 6).

The results also indicate that, compared to ventilation via polypropylene discs, convective throughflow ventilation increased leaf production and leaf growth in *Annona*; because of the increased leaf production the percentage leaf abscission was lower with the convective flow system. Also, coconut calloid was found (*a*) to contain more meristematic centres in the presence of convective-flow ventilation, and (*b*) to develop a more definite epidermal layer and a smoother surface of the nodules. Thus, this long-term calloid resembled newly formed calloid which is capable of somatic embryo and plantlet formation. The convective flow ventilation appeared to have assisted, at least partly, in moving towards the aim of obtaining vegetative propagation from 'long-term' coconut calloid.

We suggest that the beneficial effects on the *Annona* plants were probably due to the more effective removal of ethylene in the convective flow assemblies, especially since the plants similarly responded to an ethylene antagonist. However, the plants may also have benefitted from higher oxygen concentrations at night and a higher CO_2 supply during the day induced by throughflow ventilation. Similarly, the coconut calloid may have also benefitted from the removal of ethylene and from increased oxygen concentrations and the more efficient removal of CO_2.

The throughflow ventilation system described here appears to have the following advantages: (*a*) it is a simple system requiring no pumps, gas cylinders etc., (*b*) it prevents the entry of contaminants such as bacteria and fungal spores, (*c*) it will reduce CO_2 depletion in the light and its accumulation in the dark, (*d*) it will ensure near-atmospheric levels of O_2 throughout the day and night, (*e*) it flushes the system free from potentially toxic gases, e.g ethylene, and (*e*) it can eliminate the need for the use of ethylene absorbents or antagonists. With this type of system, CO_2-enrichment could easily be applied during the day for increasing photosynthesis.

While Jackson *et al.* (1991) acknowledge that the problem of ethylene accumulation can be lessened by the use of

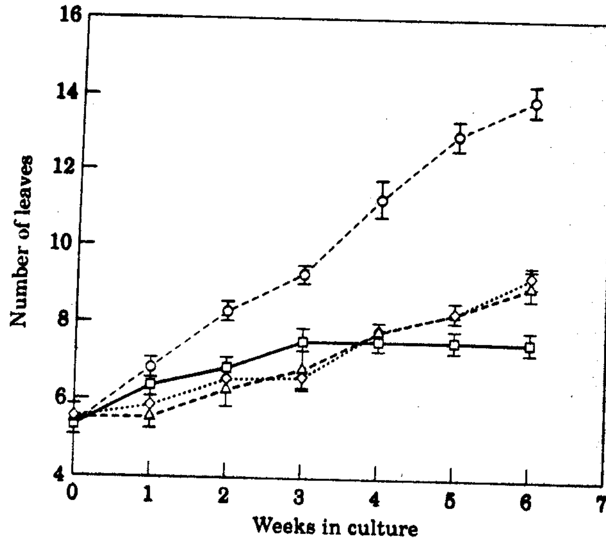

FIG. 4. Leaf production of *Annona squamosa* cuttings: the effects of the pressure-flow or the diffusive ventilating systems, with and without the ethylene antagonist silver thiosulphate (STS): 0·5 mg l^{-1}, i.e. 3·06 μmol Ag l^{-1}, in the culture medium. Means and standard errors of four replicates per treatment. Convective flow ventilation (○); convective flow ventilation+STS (△); diffusive ventilation (□); diffusive ventilation+STS (◇).

branes. Here the t_{50} for the throughflow ventilation compared to that with set A (Table 2) can be accounted for by a narrower head space in the in-flow turret and the lower RH of set B.

Effects on Annona *of different ventilation methods, with or without an ethylene antagonist*

After 6 weeks, explants grown in convective ventilation without $Ag_2S_2O_3$ in the medium, had produced at least 1·5 times more leaves than those in any other treatment (Fig. 4), and twice the number produced with diffusive ventilation without $Ag_2S_2O_3$. The leaves were larger with convective ventilation than in the other treatments, (results not shown).

For plants grown in diffusive ventilation and lacking $Ag_2S_2O_3$, the number of dropped leaves was the greatest up to week 5, but at 6 weeks the number was similar to that of the treatment with throughflow ventilation, (Fig. 5A). However, in the former treatment, where there were few leaves, almost all (98%) had dropped, whereas in the latter where leaves were more numerous because of a greater production, only approx. 50% had dropped (Fig.5B).

In treatments with either convective or diffusive ventilation with $Ag_2S_2O_3$ in the medium, the numbers of leaves produced during the experimental period were similar, and lower than those produced in convective ventilation without $Ag_2S_2O_3$ (Fig.4). Also, the numbers of leaves abscinded (Fig. 5A), and the percentages of abscissions in the two former treatments, were lower than in the other two treatments (Fig. 5B). Toxic effects of $Ag_2S_2O_3$ have been reported (Wang and Dunlap, 1990) and though smaller percentages of abscission were found in both treatments

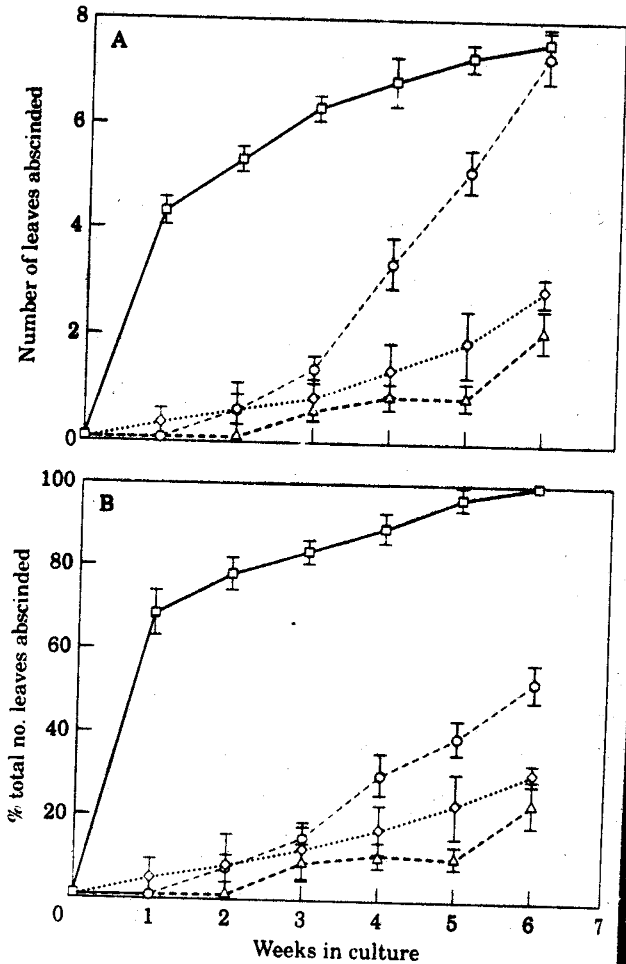

FIG. 5. *Annona squamosa* cuttings: the effects of the pressure-flow or diffusive ventilating systems, with and without the ethylene antagonist silver thiosulphate (STS) in the culture medium on (A) leaf fall, and (B) per cent leaf abscission [(number of leaves abscinded/total number of leaves) × 100]. Diffusive ventilation (□); diffusive ventilation+STS (◇); convective flow ventilation (○); convective flow ventilation+STS (△). Means and standard errors of four replicates per treament. STS at 0·5 mg l^{-1}, i.e. 3·06 μmol Ag l^{-1}.

with $Ag_2S_2O_3$, it seems possible that a toxic effect of that compound had influenced the number of leaves produced (Fig. 4).

Effects of various methods of ventilation on ethylene accumulation in culture vessels containing Annona *cuttings*

As expected, ethylene accumulated to a far greater extent in the sealed vessel compared to the ventilated ones, with mean concentrations amounting to approx. 1·9 μl l^{-1} in the former after 48 h, whereas with polypropylene capping the value was > 0·2 μl l^{-1}, and with throughflow ventilation it was negligible (Fig. 6). *Annona* has been found to be very sensitive to ethylene (Lemos and Blake, 1994), and it seems possible that the accumulation of the gas in vessels capped with polypropylene might have been sufficient to contribute to the greater incidence of leaf abscission (Fig. 5) and the smaller numbers of leaves produced (Fig. 4) compared to

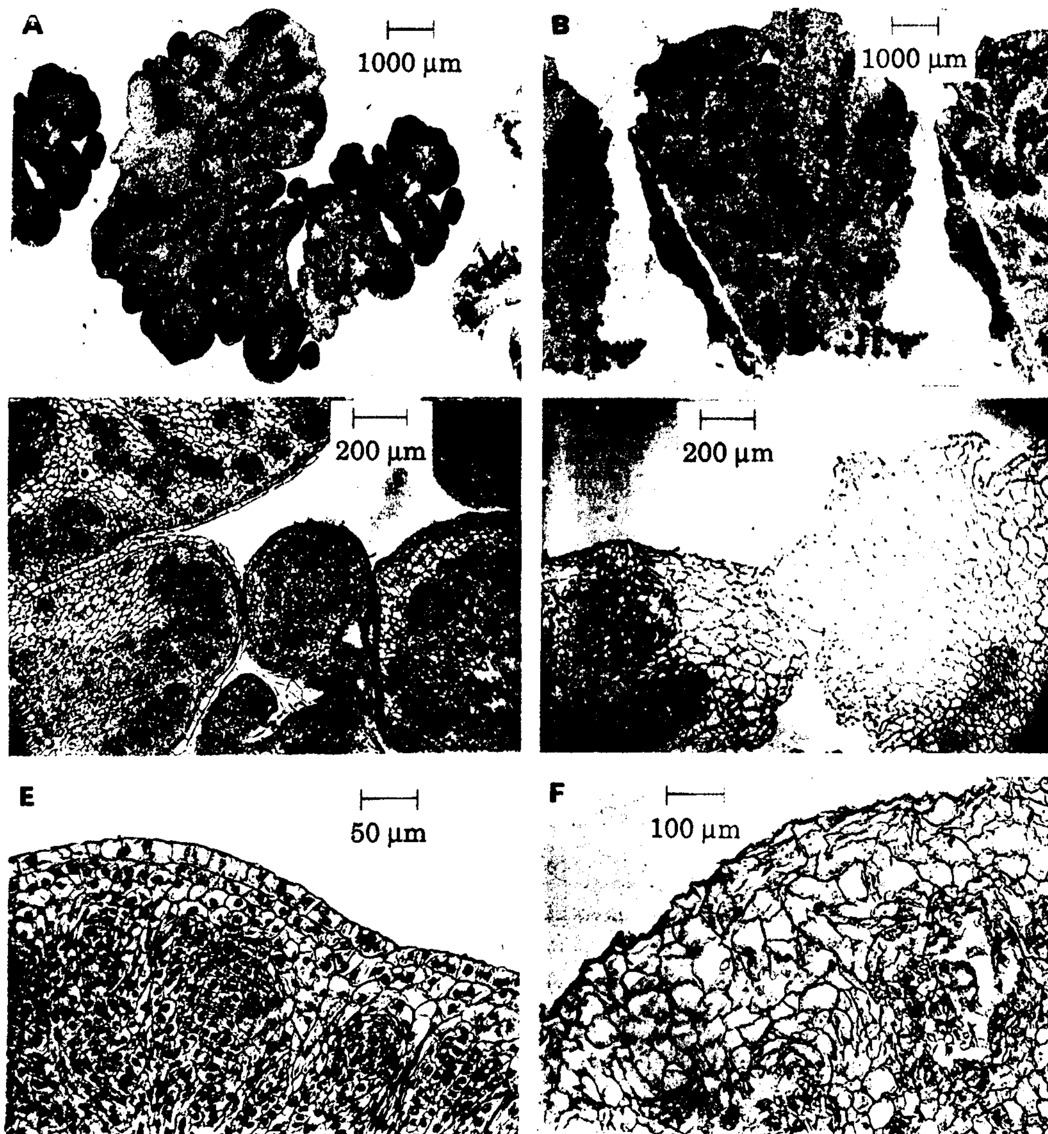

FIG. 7. Sections of wax-embedded, long-term coconut calloid of cv. Malayan Dwarf which had been grown for 20 weeks in either a sterile convective flow ventilation system (A, C, E), or a conventional culture system relying on gas diffusion through polypropylene film (B, D, F). Details as follows. A, Note the markedly convoluted shape, and profusion of discrete sub-epidermal meristematic nodules. B, Note the non-convoluted shape and fewer and less discrete meristematic nodules. C, Note the distinct well differentiated epidermal and, in places, hypodermal layers, small cells and discrete meristematic nodules. D, Note the larger cells, and less well defined epidermal layer. E, Showing similar features to (C). F, Showing similar features to (D).

larger culture vessels, the system described here makes possible the use of smaller vessels. A further possible advantage of the system is that the aerating gases are humidified, and this should help to reduce losses of water vapour from both plants and medium. Sallanon and Maziere (1992), found that low vessel and growth room humidities, by inducing high transpiration rates, produced large changes in the growth and morphology of rose plants, with decreased multiplication rates, short shoots and the cessation of the growth of the upper axillary buds. These authors concluded that the control of humidity during the multiplication phase is as important as medium composition, light and temperature parameters in the management of the plants. On the other hand, Debergh *et al.* (1992) found that the quality of *in vitro* cultured plants such as *Rosa multiflora* and *Gerbera jamesonii* could be improved by lowering the humidity of gases in the header space, compared to that developed in a closed container. They concluded that vitrification was correlated with too high a humidity in the head space. As described here the humidity-induced pressurized ventilating system results in an RH of 88–94% in the culture vessel. However, using larger membranes it is possible to create a range of controlled humidities in the culture tube of from < 70 to > 90% RH (Armstrong, Armstrong and Zobayed, unpubl. res.).

A disadvantage of the humidity-induced pressure flow

system, however, is the need to adjust daily the level of water in turret A, to maintain a high flow. The use of a constant head device connected to turret A could reduce the frequency of these adjustments. Also, if desired, much larger inflow and outflow chambers, with large Nuclepore filter membranes could be employed to create more rapid ventilation in culture vessels containing several plants, or to supply fresh air to several small vessels in parallel. We have recently developed a system combining the inflow and outflow turrets in one unit, which sustains effective humidification for the inflow of air for at least a week without attention (Armstrong, Armstrong and Zobayed, unpubl. res.).

ACKNOWLEDGEMENTS

We thank Mr Mike Bailey for constructing the convective flow assemblies, Mrs Margaret Huffey for help with the GLC, and Mr Richard Holt for photographic assistance, all of the University of Hull. Also, we are grateful to Dr Ken Giller and Dr George Cadisch who kindly provided equipment and facilities for gas chromatography in the Dept. of Biochemistry and Biological Sciences at Wye College. We also thank Dr Janet Blake for critically reading the manuscript. The work at Wye College was supported by the Overseas Development Administration research scheme R4658 (SHFWJ) and by the Brazilian Conselho Nacional de Desenvolvimento Cientifico e Tecnologico–CNPq (EEPL).

LITERATURE CITED

Adkins SW. 1992. Cereal callus cultures: control of headspace gases can optimise the conditions for callus proliferation. *Australian Journal of Botany* **40**: 737–749.

Armstrong J. 1992. *Pathways and mechanisms of aeration in* Phragmites australis. PhD Thesis, University of Hull, U.K.

Armstrong J, Armstrong W. 1990. Light-enhanced convective through-flow increases oxygenation in rhizomes and rhizosphere of *Phragmites australis* (Cav.) Trin. ex Steud. *New Phytologist* **114**: 121–128.

Armstrong J, Armstrong W. 1994. A physical model involving nuclepore membranes to investigate the mechanism of humidity-induced convection in *Phragmites australis*. *Proceedings of the Royal Society of Edinburgh* **102B**: 529–540

Armstrong W, Armstrong J, Beckett PM. 1996*a*. Pressurised aeration in wetland macrophytes: some theoretical aspects of humidity-induced convection and thermal transpiration. *Folia Geobotanica et Phytotaxonomica* **31**: 25–36.

Armstrong W, Armstrong J, Beckett PM. 1996*b*. Pressurised ventilation in emergent macrophytes: the mechanism and mathematical modelling of humidity-induced convection. *Aquatic Botany* (in press).

Armstrong J, Armstrong W. 1996*c*. *Ventilation apparatus and system.* University of Hull UK Patent No. GB227505B. The Patent Office, London. Publication number (August 1994) 2275052A.

Armstrong W, Armstrong J, Beckett PM, Justin SHFW. 1990. Convective gas-flows in wetland plant aeration. In: Jackson MB, Davies DD, Lambers H, eds. *Plant life under oxygen stress.* The Hague: SPB Academic Publishing bv, 283–302.

Blake J, Hornung R. 1994. Somatic embryogenesis in coconut. In: Jain SM, ed. *Somatic embryogenesis in woody plants.* Dordrecht, The Netherlands: (in press).

Blake J, Wilson N. 1990. Problems of somatic embryogenesis in coconut (*Cocos nucifera* L.). In: *Abstracts of the VIIth International Congress of Plant Tissue and Cell Culture.* Amsterdam 24–29 June 1990. International Association of Plant Tissue Culture. Abstract B4-19, 245.

Brackpool AL, Branton RL, Blake J. 1986. Regeneration in palms. In: Vasil IK, ed. *Cell culture and somatic cell genetics of plants.* London: Academic Press, 207–222.

Branton RL, Blake J. 1983*a*. Development of organised structures in callus derived from explants of *Cocos nucifiora* L. *Annals of Botany* **52**: 673–678.

Branton RL, Blake J. 1983*b*. A lovely clone of coconuts. *New Scientist* **98**: 554–557.

Branton RL, Blake J. 1986. Clonal propagation of coconut palm. In: Pushparajah E, Chew Poh Soon, eds. *Cocoa and coconuts: progress and outlook.* Kuala Lumpur: Incorporated Society of Planters. 771–780.

Brix H, Sorrell BK, Orr PT. 1992. Internal pressurisation and convective gas-flow in some emergent freshwater macrophytes. *Limnology and Oceanography* **37**: 1420–1433.

Buddendorf-Joosten JMC, Woltering EJ. 1996. Controlling the gaseous composition *in vitro*–description of a flow system and effects of the different gaseous components on *in vitro* growth of potato plantlets. *Scientia Horticulturae* **65**: 11–23.

Dacey J. W. H. 1981. Pressurised ventilation in the yellow water-lily. *Ecology* **62**: 1137–1147.

Debergh PC, De Meester J, De Riek J, Gillis S, Huylenbroeck J van. 1992. Ecological and physiological aspects of tissue-cultured plants. *Acta Botanica Neerlandica* **41**: 417–423.

De Proft MP, Maene LJ, Debergh PC. 1985. Carbon dioxide and ethylene evolution in the culture atmosphere of *Magnolia* cultured *in vitro*. *Physiologia Plantarum* **65**: 375–379.

Fremond Y, Ziller R, de Nuce de Lamothe M. 1966. *The coconut palm.* International Potash Institute (Publ).

Jackson MB, Abbott AJ, Belcher AR, Hall KC. 1987. Gas exchange in plant tissue cultures. In: Jackson MB, Mantell S, Blake J, eds. *Advances in the chemical manipulation of plant tissue cultures.* BPGRG Monograph 16. Bristol: British Plant Growth Regulator Group, 57–71.

Jackson MB, Abbott AJ, Belcher AR, Hall KC, Butler R, Cameron J. 1991. Ventilation in plant tissue culture and effects of poor aeration on ethylene and carbon dioxide accumulation, oxygen depletion and explant development. *Annals of Botany* **67**: 229–237.

Jackson MB, Belcher AR, Brain P. 1994. Measuring shortcomings in tissue culture aeration and their consequences for explant development. In: Lumsden PJ, Nicholas JR, Davies WJ, eds. *Physiology, growth and development of plants in culture.* Dordrecht, The Netherlands: Kluwer Academic, 191–203.

Kozai T, Kitaya Y, Kubota C. 1995. *Collected papers on environmental control in micropropagation*, Vol. 3 (1994–1995). Genhua Niu, ed. Laboratory of Environmental Control Engineering, Faculty of Horticulture, Chiba University, Chiba 271, Japan.

Kozai T, Kubota C, Nakayama M. 1989. Net photosynthetic rates of plantlets *in vitro* under natural and forced ventilation conditions. *Annual Meeting, Japanese Society of Horticultural Science.* 250–251.

Lemos EEP, Blake J. 1994. Leaf abscission in micropropagated sugar apple (*Annona squamosa* L.). In: Lumsden PJ, Nicholas JR, Davies WJ, eds. *Physiology, growth and development of plants in culture.* Dordrecht, The Netherlands: Kluwer Academic, 227–232.

Murashige T, Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15**: 473–497.

Righetti B, Facini O. 1992. Headspace gas composition in four *Prunus avium* cultivars with differing photosynthetic capabilities. In vitro *cellular developmental biology*, (Tissue Culture Association) **28P**: 179–182.

Righetti B, Magnanini E, Infante R. 1990. Ethylene, ethanol, acetaldehyde and carbon dioxide release by *Prunus avium* shoot cultures. *Physiologia Plantarum* **78**: 507–510.

Righetti B, Magnanini E, Maccaferri M. 1987. Ethylene and other volatile substances produced by *in vitro* cultured *Prunus avium*. *Acta Hortanica* **227**: 402–404.

Rossetto M, Dixon KW, Bunn E. 1992. Aeration: a simple method of control of vitrification and improved *in vitro* culture of rare Australian plants. In vitro *cellular developmental biology*, (Tissue Culture Association) **28P**: 192–196.

Sallanon H, Maziere Y. 1992. Influence of growth room and vessel humidity on the *in vitro* development of rose plants. *Plant Cell, Tissue and Organ Culture* **30**: 121–125.

Thomas D, Des S, Murashige T. 1979. Volatile emissions of plant tissue cultures. I. Identification of the major components. *In Vitro* **15**: 654–658.

Wang Y-T, Dunlap JR. 1990. Leaf abscission in *Radermachera sinica* in response to ethylene and silver thiosulphate. *Horticultural Science* **25**: 233.

Woltering EJ. 1986. Ethylene and carbon dioxide accumulation within various tissue culture systems. *Acta Botanica Neerlandica* **35**: 50 (Abstract).

Yue D, Gosselin A, Desjardins Y. 1993. Effects of forced ventilation at different relative humidities on growth, photosynthesis and transpiration of geranium plantlets *in vitro*. *Canadian Journal of Plant Science* **73**: 249–256.

Zobayed, SMA. 1996. *The use of a natural pressorized forced ventilation in plant micropropagation*. PhD Thesis, University of Hull.