THE UNIVERSITY OF HULL

# THE USE OF NATURAL PRESSURISED FORCED VENTILATION IN PLANT MICROPROPAGATION

**being a Thesis submitted for the**
**Degree of Doctor of Philosophy**

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**by**

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**Summary of the thesis for the PhD degree**
**by Sayed Md. Akhter Zobayed**
**on**
**"The use of natural pressurised Forced Ventilation in Plant Micropropagation"**

A new, uncomplicated system for the forced ventilation of plants and cultures has been investigated in terms of both its efficiency of ventilation and its effects on the growth and physiology of various plant species, including cauliflower, tobacco, *Annona* (custard apple) and potato.

This new system, which has no moving parts or artificial energy requirement, provides a sustained, pressurised stream of sterile, humidified air (RH = 70-94%) driven by humidity-induced diffusion. This process depends upon the maintenance of a gradient of water vapour across a microporous partition for inducing the diffusion of air into the apparatus. Flows up to 5 $cm^3$ $min^{-1}$ can be produced and the atmosphere in a 60 $cm^3$ culture vessel can be renewed every 12 min Compared to the standard conventional diffusive method of ventilation, *e.g.* by capping the vessel with a polypropylene disc, this new system has proved to be 18X more efficient in removing accumulated ethylene and in keeping $CO_2$ and $O_2$ levels in culture vessels close to atmospheric.

This forced ventilation system has also been shown to be very effective in the *in vitro* cultivation of seedlings or cuttings of cauliflower, tobacco, *Annona* and potato for improving growth and preventing symptoms of vitrification such as leaf epinasty, reduction of leaf area and production of abnormal stomata. In potato cuttings the induction and production of microtubers have been promoted and the growth of abnormal callus prevented. In *Annona* cuttings flower bud production, leaf and shoot growth and micropropagation have been promoted and leaf and flower bud abscission have been reduced. In cauliflower, tobacco and *Annona* the leaf chlorophyll contents, rates of photosynthesis and yields were improved by this forced ventilation. These beneficial effects have been variously attributed to the efficient removal of ethylene, the maintenance of near to atmospheric levels of $CO_2$ and $O_2$ by day and night and to the reduction of humidity levels in the vessels to below 100% RH.

It is hoped that this new ventilation system, which is comparatively inexpensive and requires very little maintenance might have some useful applications in the field of tissue culture and perhaps particularly in developing countries.

## ABBREVIATIONS

| | |
|---|---|
| ACC | 1- aminocyclopropane-1-carboxylic acid |
| AOA | aminooxyacetic acid |
| AVG | aminoethoxyvinylglycine |
| BAP | 6-benzyl amino purine |
| CCCP | carbonyl cyanide *m*-chlorophenylhydrazone |
| CH | casein hydrolysate |
| $D_k$ | Knudsen diffusion coefficients |
| DNP | 2,4-dinitrophenol |
| $D_o$ | mutual diffusivity |
| HIC | humidity-induced convection |
| IAA | indole-3-acetic acid |
| IBA | indole-3-butyric acid |
| $J_c$ | diffusion rate |
| MPD | membrane pore diameter (μm) |
| MPDi | inflow membrane pore diameter (μm) |
| MPDo | outflow membrane pore diameter (μm) |
| MS | Murashige and Skooge (1962) medium |
| NAA | α naphthalene acetic acid |
| PAR | photosynthetically-active radiation (μmol $m^{-2}$ $s^{-1}$) |
| RH | relative humidity (%) |
| SAM | *S*-adenosylmethionine |
| SEM | scanning electron microscopy |
| W | distance between water surface and membrane (mm) |
| Wr | distance between water-reservoir surface and membrane (mm) |
| Wo | distance between water-saturated Oasis and membrane (mm) |
| w/v | mass to volume ratio |
| $\Delta P_d$ | dynamic pressure differential |
| $\Delta P_s$ | static pressure differential |
| $\Delta C$ | concentration difference |
| Plantlet | *In vitro*-grown shoot with root system |
| Cutting | *In vitro*-grown shoot without any root system |
| FF-ventilation | Fast flow forced ventilation (Flow = 3.5 - 5.0 $cm^3$ $min^{-1}$) |
| SF-ventilation | Slow flow forced ventilation (Flow = 1.0 - 2.0 $cm^3$ $min^{-1}$) |

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# CHAPTER - I

## GENERAL INTRODUCTION

Micropropagation is a particularly useful research tool and is a popular and expanding area for the commercial production of plants *in vitro*. The major goal of commercial micropropagation is to achieve, in as reduced a time period and at as low a cost as possible, a large number of genetically identical, physiologically uniform, and developmentally normal plantlets, preferably with high photosynthetic or photoautotrophic potential (utilizing $CO_2$ in the air as the carbon source) and the ability to survive comparatively harsh *ex vitro* conditions (Jeong, Fujiwara and Kozai 1995). However, the commercial application of plant micropropagation is still limited, mainly due to high production costs, low growth rates, and ultimately to poor percentage survival during the acclimatization period.

Growth and development of plantlets or explants produced *in vitro* depend not only on the composition of the nutrient medium but may also be affected by the composition of the gaseous atmosphere (Jackson *et al.* 1987; Blazková *et al.* 1989). Recently, extensive research and effort have been focused in areas such as automation and robotization of micropropagation processes (Aitken-Christie and Jones 1987; Johnson 1989; Aitken-Christie 1991; Brown 1992; Jeong 1992), plant image recognition and processing, microcutting growth, transplanting (McElroy and Brown 1992), handling and manipulation of culture vessels (Gautz and Wong 1992) and modification of nutritional components and growth regulators in the medium. However, the various aspects of the gaseous atmosphere of the culture vessels have received relatively little attention (Buddendorf-Joosten and Woltering 1994).

The conventional protective conditions under which plant materials are grown to prevent microbial contamination and retard desiccation of the tissues and the nutrient medium can cause unintentional restriction of the exchange of gases between the vessel atmosphere and the outside air (Buddendorf-Joosten and Woltering 1994). Sealing materials

normally used *in vitro* are cotton plugs, cellulose stoppers, screw caps, aluminium foils, transparent films such as polypropylene, parafilm, cling film etc. Therefore, the gaseous environment *in vitro* is often abnormal when compared with the *ex vitro* environment. The major characteristics of the gaseous environment *in vitro* in conventional tissue culture systems are high relative humidity, large diurnal fluctuation in $CO_2$ concentration and the accumulation of ethylene and other toxic substances (Kozai *et al.* 1992). As a consequence the photosynthesis, transpiration, and uptake of water, nutrients and $CO_2$ can be suppressed and dark respiration enhanced, resulting in poor growth (Jeong, Fujiwara, and Kozai 1995) and physiological and morphological disorders of the cultured plantlets (Debergh and Maene 1984; also Table 1.01 and Fig. 1.01) including undesirable morphogenetic changes (Table 1.01; Fig. 1.01) that are varied and species dependant (Jackson *et al.* 1987).

For healthy growth, all parts of a plant must exchange their internal gases readily with those in the surrounding air. Nevertheless, in conventional tissue culture systems the exchange of gases between tissues and the air is frequently restricted and often severely so (Jackson *et al.* 1987). Many plant species when grown *in vitro* release a variety of substances which may accumulate and have significant effects on growth and development (Heyser and Mott 1980). The most widely studied gaseous product from cultures is ethylene (Gamborg and LaRue 1968, Huxter, Reid and Thorpe 1979). This gas is associated with various physiological responses such as induced epinasty (Crocker, Zimmerman and Hitchcock 1932), leaf abscission (Burg 1968, Lemos and Blake 1994, Armstrong *et al.* 1996), flower and fruit maturation and senescence (Yang and Hoffman 1984; Reid 1987) during plant growth and development. A number of tissue culture stages can be affected by ethylene and result in, for example, poor cell differentiation (Miller and Roberts, 1984), an absence of somatic embryogenesis (Meijer and Brown, 1988; Purnhauser *et al.* 1987; Roustan, Latche, and Fallot 1990; Wochok and Wetherell 1971), reduced shoot height and leaf area (Jackson *et al.* 1987 and 1991) and poor callus proliferation and growth (Adkins,

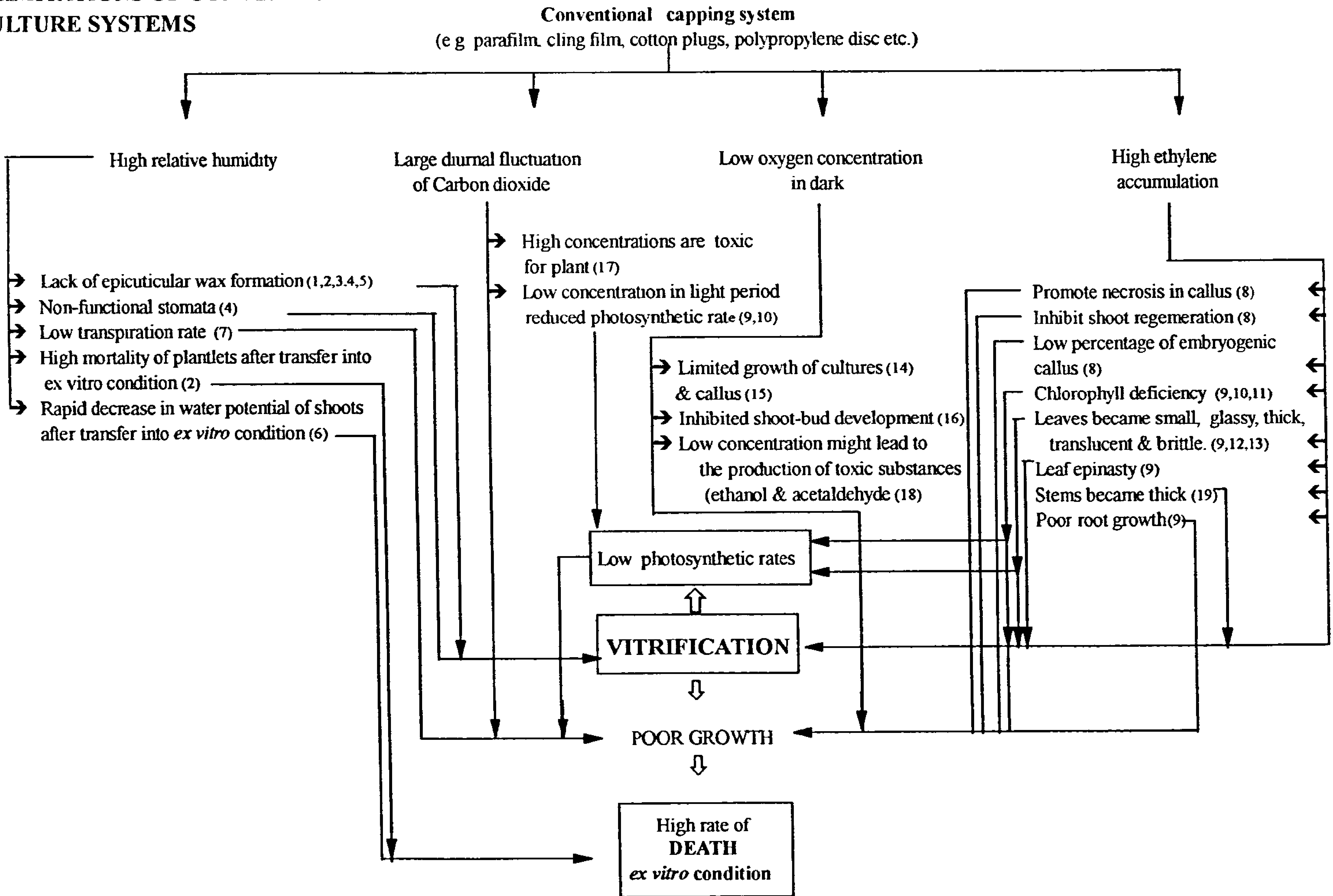
**TABLE 1.01 : Effects of ethylene on cultured tissues / explants and the promotive effect of ethylene inhibitors on growth and morphogenesis of plant cells and tissues *in vitro*.**

| Plant species | Tissue/Explant used | Effect of Ethylene on cultured tissue | Amount of ethylene | Ethylene inhibitor | Effects of ethylene inhibitors | References |
|---|---|---|---|---|---|---|
| **Monocotyledons** | | | | | | |
| *Oryza sativa* | Callus | Reduces growth & promotes necrosis | 500 nl/w (IR42 variety; at 35°C; light | AVG<br>Silver nitrate | Increased callus growth (100% with AVG & 60% with $AgNO_3$ | Adkins *et al* 1993 |
| *Triticum aestivum* | Immature embryo derived callus | Inhibits shoot regeneration | -- | Silver nitrate | Enhancement of shoot regeneration | He, Yang and Scott 1989 |
| *Zea mays* | Immature embryos | Reduces type II callus production and 25% embryos produce callus | 115 - 370 nl/g/h (B73XA1188 variety) | Silver nitrate | Promotes callus production & 80% embryos produce callus. | Songstad *et al* 1989 |
| **Dicotyledons** | | | | | | |
| *Acer pseudoplatanus* | Suspension culture | - | 1.5nmoles/g/h(no additive)<br>4.22 nmoles/g/h(Fusicoccin) | AVG, cobalt ion | Ethylene production was strongly inhibited | Malerba *et al* 1995 |
| *Arabidopsis* spp | Roots | Reduces shoot regeneration | -- | Silver nitrate (25 mg/l) | Increases the number of shoots | Márton & Browse 1991 |
| *Brassica* spp | Cotyledons & Hypocotyl | Poor regeneration of shoots (10-20%) | 2.0-2.5 µl/h (14 d culture) | AVG | Shoot regeneration increased progressively (upto 70-90%) | Chi *et al* 1990 |
| *Brassica oleracea* (Brussels sprouts) | Anther | Low percentage of embryogenic callus | -- | Silver nitrate | Increases embryo production and embryo yield | Biddington, Sutherland & Robinson 1988 |
| *Brassica oleracea* & *Brassica napus* | Hypocotyl | -- | -- | Silver nitrate (2-5 mg/l) | Ethylene inhibitor is an absolute pre-requisite to obtain transformed shoots | De Block, Brouwer & Tenning1989 |
| *Daucus carota* | Hypocotyl derived cell suspension | Inhibits embryo formation | 1.5-1.7 nl/h/g (Fresh wt.) (within 24 h) | $CoCl_2$ (10-50 µM)<br>$NiCl_2$ (20-100µM) | Enhanced ability to develop embryos-9N/m -50µm cobalt;45N/ml-100µm nickel chloride | Roustan *et al* 1989 |
| *Helianthus annuus* | Cotyledons | Poor shoot regeneration (1.2%) | 36 µl/h/g (5th day of culture) | $CoCl_2$<br>Silver nitrate | Enhanced shoot regeneration (30% with 20 µm CoCl2 & 25% with 10-25µm AgNO3) | Chraibi *et al* 1991 |
| *Helianthus annuus* | Hypocotyl derived callus | Poor somatic embryos (0.3±0.2 embryo/callus piece) | 115±26 pmol/8h-in light<br>92±30 pmol/8h-in dark | AVG (50 µM) | Increase number of somatic embryos (1.5±.4 embryo/callus) | Robinson & Adams 1987 |

**Table 1.01 ( cont.)**

| Plant species | Tissue/Explant used | Effect of Ethylene on cultured tissue | Amount of ethylene | Ethylene inhibitor | Effects of ethylene inhibitors | References |
|---|---|---|---|---|---|---|
| *Hevea brasiliensis* | Callus | Enhances browning of callus & inhibits the embryogenic tissue formation (1% without AOA ) | -- | AOA (250 µM) | Increase embryogenic tissue formation (34% with AOA treatment) | Housti, Coupe & d'Auzac 1992 |
| *Hordeum vulgare* cultivar-Klages | Anther | Optimum level is important for embryogenesis but higher concentrations are deleterious | 5.6nl/h/g-anther culture at late uninucleate stage; after 2 h incubation | Putrescine n-pryopyl gallate | Increased embryogenesis | Cho and Kasha 1989 |
| *Lycopersicon pennellii* | Shoot derived protoplast | Low yield ($1.8X10^6$ /gFW pps-without STS), low plating efficiency (1%) & low regeneration | 1699nl/gFW- enzymatically (7% cellulysin) - released protoplast | Silver thiosulphate 2-5 mg/l | Improved protoplast yield($3.1X\ 10^6$ /gFW pps),viability higher plating efficiency(4%) & high regeneration capacity | Rethmeier *et al* 1991 |
| *Nicotiana tabacum* | Callus | Small amounts of ethylene speed up the rate of shoot emergence but high amounts have the opposite effect | 750 nl/callus piece/day (FW=1.5g;in dark condition | Silver nitrate Mercuric perchlorate | No significant effect on growth | Huxter, Reid & Thorpe 1979 |
| *Pinus radiata* | Cotyledons | Excessive accumulation ethylene causes some degree of dedifferentiation | 25.3 µl/l (flask capped with serum caps & replaced with foam bungs on 21st days) | -- | -- | Kumar, Reid & Thorpe 1987 |
| *Picea glauca* (White spruce) | Somatic embryo | Inhibits somatic embryo maturation | 3.5-4.5 nl/g FW tissue/h (- no ABA added) | ethapon, silver nitrate, cobalt chloride, & AVG | Presence of AVG or silver improved the quality of the embryo by preventing intercellular space formation in the shoot pole of the embryo | Kong & Yeung 1994 |
| *Solanum carolinense* | Pollen | Implicate embryogenesis | 1-1.5% nl/24h/culture (4-8th days of culture | cobalt chloride | Reduced the amount of ethylene but did not eliminate the embryogenesis | Reynolds 1987 |
| *Solanum tuberosum* | Anther | Inhibits embryoid formation | -- | ascorbic acid L-cysteine | Prevents browning of anther culture & stimulate embryogenesis | |
| | | | | Silver nitrate, polyamines n-propylgallate spermidine & putrescine | Promoted embryogenesis | Tiainen 1992 |
| | | | | Glutathione | Slightly increase embryoid yield | |
| *Solanum tuberosum* | Shoot | Production of short swollen stems, hooked stem apex, vestigial stem leaves and small root systems | 122.5µl/l (100 ml flasks capped with Suba-seal) | Mercuric perchlorate | Prevents swelling & shortening of the shoot. normal stem tip, root & leaf morphology | Jackson *et al* 1987 |

FIG. 1.01. LIMITATIONS OF CONVENTIONAL TISSUE CULTURE SYSTEMS




(1) Grout & Aston 1977; (2) Sutter & Langhans 1979; (3) Wardl. Quinlan & Simpkins 1979; (4) Brainered and Fuchigami 1982; (5) Brainered and Fuchigami 1981; (6) Fujiwara, Aitken-Christie and Kozai 1993; (7) Kozai and Smith (1995). (8) table 1 01; (9) Chapter-IV; (10)Chapter- V. (11) Chapter VII, (12) Ziv 1991b; (13) Paque and Boxus 1987; (14)Tate & Payne 1991; (15) Adkins. Shiraishi & McComb 1990; (16) Kumar. Reid & *Thorpe 1987; (17) Buddendorf-Joosten & Woltering 1994*. (18) Adkins 1992; (19) Chapter-VIII

Shiraishi, and McComb 1990). Some of these effects on cultured tissue are summarised in Table 1.01.

The accumulation of ethylene in the culture vessels is also responsible for the vitrification of a number of species (Jackson *et al.* 1991). Symptoms of vitrification include chlorophyll deficiency, cell hyperhydricity, hypolignification, reduced deposition of epicuticular waxes, and changes in enzymatic activity and protein synthesis (Ziv 1991a,b). Vitrified propagules of plants appear "glassy", with thick, translucent, and brittle leaves and showing excessive basal growth and callus formation (Paque and Boxus 1987; Ziv 1991b). As a result, such plantlets desiccate rapidly and die when transferred to soil. Vitrification is considered to be a serious problem in micropropagation of many species (Williams and Taji 1991).

The pathway of ethylene biosynthesis and the mechanism of its biological activity are not yet fully understood, but for many years it has been known that methionine is the principal biological precursor for ethylene biosynthesis (Lieberman 1979). The tracer studies of Adams and Yang (1977) showed that *S*-adenosylmethionine (SAM) is an intermediate between methionine and ethylene. Adams and Yang (1979) identified l-aminocyclopropane-l-carboxylic acid (ACC) as the immediate precursor and thus proposed a pathway for ethylene biosynthesis from methionine to SAM to ACC to ethylene (Fig 1.02). ACC is then rapidly converted to ethylene in air and this step requires oxygen. However, the oxygen-dependent conversion of ACC to ethylene still remains to be explained at $CO_2$ concentrations higher than 20% and when fermentation products are formed (Righetti 1990). $CO_2$ may also play a role in the conversion of ACC to ethylene (Philosoph-Hadas, Aharoni, and Yang 1986).

The biosynthesis of ethylene from methionine is enhanced by carbohydrates (e.g sucrose - used in culture media) (Philosoph-Hadas, Meir and Aharoni 1985), light, cytokinins (Lieberman 1979) and $CO_2$ (Kumar, Reid and Thorpe 1987). Photosynthesis and respiration are the main processes modulating the concentrations of three of the most

important components of the culture vessel atmosphere: $CO_2$, $O_2$, $C_2H_4$. These processes depend in turn on light intensity and quality, photoperiod, redox potential of the liquid-solid substrate, the partial pressures affecting chemical interaction, and enzymatic equilibria during incubation (Righetti and Facini 1992). Gaseous pollutants may also be introduced into the atmosphere inside culture vessels during transplanting operations (Righetti *et al.* 1990). Agar, the gelling agent in the culture medium, itself can discharge ethylene (Mensuari-Sodi, Panizza and Tognoni 1992).

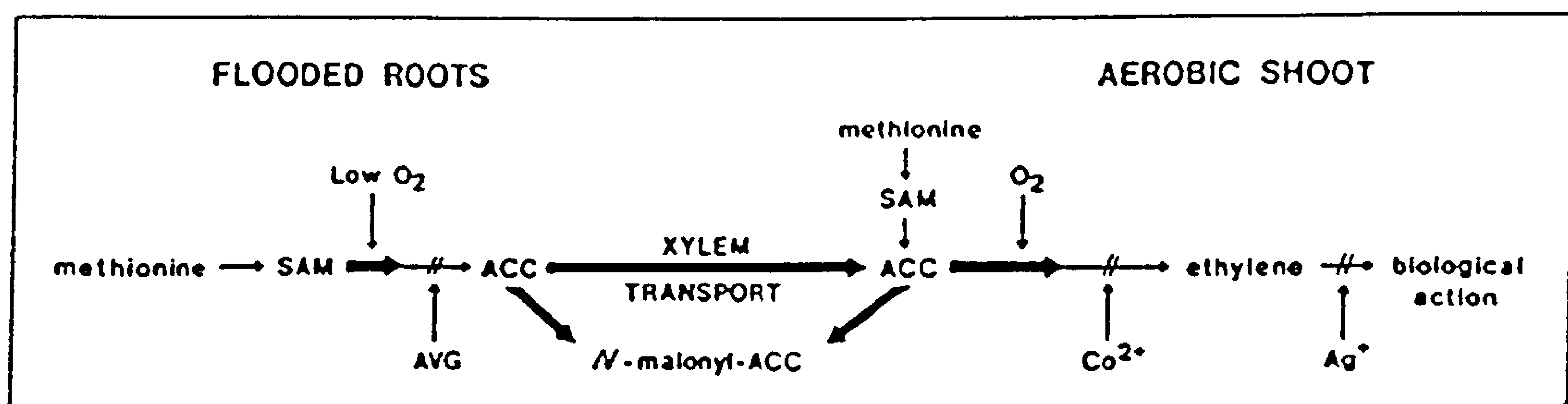



**Fig 1.02.** **Scheme for ethylene physiology of flooded plants. The heavy arrows indicate that the process is promoted, whereas the diagonal lines signify inhibition. ACC, l-Aminocyclopropane-l-carboxylic acid; $Ag^+$, silver ion; AVG, aminoethoxyvinylglycine; $Co^{2+}$, cobaltous ion; SAM, *S*-adenosylmethionine (From - Reid and Bradford 1984).**

The effects of ethylene can often be reduced by the addition of $AgNO_3$ to the culture medium or of Norbornadiene to the atmosphere. Other ethylene 'inhibitors' are salicylic acid, analogues of ACC e.g. $\alpha$-aminoisobutyric acid, pyridoxyl enzyme inhibitors such as AVG, AOA, inorganic ions $Co^{2+}$ and $Ni^{2+}$, free radical scavengers e.g. *n*-propyl gallate, polyamines i.e. spermidine and spermine, and membrane disrupting agents e.g. DNP and CCCP etc, but how these inhibitors reduce or deactivate ethylene is not always fully understood. Bradford and Yang (1981) and Bradford, Hsiao, and Yang (1982) reported that ACC production could be inhibited by AVG and the conversion of ACC to ethylene could

be inhibited by $Co^{2+}$(Fig 1.02). Chi and Pua (1989) reported that the inhibitory effect of $Ag^+$ is believed to be due to an interference with the binding of ethylene. Adams and Yang (1977) reported that the receptor site of ethylene is believed to contain $Cu^+$, and $Ag^+$ was thought to alter ethylene action by substituting for $Cu^+$ at the receptor site. AVG strongly inhibited the conversion of methionine to ACC (Fig. 1.02), but it did not block the conversion of ACC to ethylene. That is AVG inhibits the conversion of SAM to ACC and the conversion is mediated by a pyridoxal enzyme (Adams and Yang 1977). AOA is another well-known inhibitor of the pyridoxal enzyme (Adams and Yang 1979, Table 1.01) which also inhibits the conversion of SAM to ACC and thus inhibits ethylene biosynthesis. Cobalt ($Co^{2+}$) and nickel ($Ni^{2+}$) are both commonly used as ethylene inhibitors (Table 1.01). Yu and Yang (1979) demonstrated that $Co^{2+}$ inhibits ethylene biosynthesis by inhibiting the conversion of ACC to ethylene. Bradford, Hsiao, and Yang (1982) reported that $Co^{2+}$ effectively inhibits ethylene production without affecting the increase in ACC level in the tomato shoot. Cobalt, in the form of $CoCl_2$, can be used as an inhibitor of ethylene production (Table 1.01).

Free radical inhibitors such as *n*-propyl gallate are known to inhibit ethylene production (Table 1.01). Konze *et al.* (1980) and Apelbaum *et al.* (1981) indicate that it inhibits the conversion of ACC to ethylene, which is sensitive to free radical scavengers.

Recently polyamines have been used as ethylene inhibitors both *in vivo* (Shih *et al.* 1982) and *in vitro* (Table 1.01). Yang and Hoffman (1984) demonstrated that the biosynthesis of ethylene and polyamines share a common precursor, SAM, and thus ethylene biosynthesis is inhibited by polyamines. Suttle (1981) found that Spermidine inhibits ethylene production (also shown in Table 1.01) by inhibiting the conversion of ACC to ethylene in Soyabean hypocotyls. Low concentrations of DNP inhibits ethylene biosynthesis by blocking the conversion of ACC to ethylene without affecting the conversion of methionine to SAM (Yu and Yang 1980). L-aminoisobutyric acid acts as structural analogue of ACC and thus inhibits ethylene production.

Large amounts of carbon dioxide have been measured in many species grown *in vitro* and often high concentrations are found in association with ethylene (Zobel 1987) Where cultures are grown under a day:night regime, carbon dioxide concentrations fluctuate due to the respiration and photosynthesis of the plants. During the dark period, due to respiration, $CO_2$ concentrations increase and during the light period the photosynthetic activity of chlorophyllous plantlets results in a decline in $CO_2$ levels. The enhancement of the $CO_2$ concentration in darkness has been reported by many authors (De Proft, Maene and Debergh 1985; Fujiwara, Kozai and Watanabe 1988; Jackson *et al.* 1991). However, the concentrations of $CO_2$ very much depend upon the ways in which the culture vessels are sealed. Jackson *et al.* (1991) demonstrated that *Ficus lyrata* cultures with loose, intermediate and tightly sealed vessels, contained respectively 0.5. 3.4 and 8.5% $CO_2$ in the dark period.

Depletions of the $CO_2$ concentration during the photoperiod were also reported by Desjardins *et al.* (1988), Kozai *et al.* (1987), Kozai and Iwanami (1988) and Solárová *et al.* (1989). In sealed conditions the concentration may drop to levels that are generally considered to be limiting (Buddendorf-Joosten and Woltering 1994). However, others have demonstrated an enhancement of $CO_2$ concentration in the culture atmosphere in the light. Woltering (1989) found 1.3% and 13% $CO_2$ with *Gerbera jasmesonii* in semi-closed and tightly sealed containers respectively. Righetti, Magnanini and Maccaferri (1988) found 20% $CO_2$ in *Prunus* shoot cultures grown in the light in (probably sealed) jars. Jackson *et al.* (1991) also reported 8.5% $CO_2$ in the dark in sealed vessels containing *Ficus lyrata* and this decreased to 0.2% and 1% at the end of the light period with loose and intermediate sealing of the culture vessels respectively. These higher $CO_2$ concentrations (>1%) are generally considered to be toxic for plants (Buddendorf-Joosten and Woltering 1994).

Generally, with increasing $CO_2$ concentrations in the culture vessels in darkness, a comparable decrease in oxygen can be expected (Buddendorf-Joosten and Woltering 1994). The production of ethylene may also accompany the depletion of oxygen in the culture head-

space. Only a few studies have been performed on the measurement of oxygen concentrations in the head-spaces of culture vessels. Doi, Oda, and Asahira (1989) found that in culture vessels containing either *Caladium bicolor* (C-3 plant) or *Dendrobium phalaenopsis* (CAM plantlets) $O_2$ concentrations during the dark period decreased in accordance with an increase in $CO_2$ concentration. During the dark period, the decrease in $O_2$ concentration was approximately equal to the increase in $CO_2$ concentration in some plantlets like *Caladium bicolor*. However, with others, (like *Dendrobium phalaenopsis* plantlets), the decrease in $O_2$ concentration was markedly larger than the increase in $CO_2$ concentration (Fujiwara and Kozai 1995). Righetti and Facini (1992) investigated the time course of $O_2$ concentration for 30 days in air-tight jars containing *Prunus avium* shoots, and showed that a large decrease in $O_2$ concentration was accompanied by increases in both $CO_2$ and ethylene concentrations.

The availability of oxygen in any tissue culture system is very important for respiration and subsequent tissue growth (Adkins 1992). Oxygen availability has limited the growth of cell cultures such as *Catharanthus roseus* (Tate and Payne 1991) and rice callus (Adkins, Shiraishi and McComb 1990). Kumar, Reid and Thorpe (1987) showed that in the absence of oxygen excised cotyledons *Pinus radiata* failed to develop shoot buds. Low oxygen levels might lead to the production of toxic compounds such as ethanol and acetaldehyde (Adkins 1992).

Other volatile substances released *in vitro* are ethane, ethanol, methane, acetylene and acetaldehyde (Thomas and Murashige 1979a,b). Rice (*Oryza sativa* L.) callus culture modified the atmosphere of the culture vessel by producing carbon dioxide, ethylene and ethanol, while utilizing oxygen (Adkins, Shiraishi and McComb 1990). These changes in the gaseous atmosphere of the culture vessel can suppress the growth of callus and promote necrosis (Adkins 1992).

Relative humidity in the culture vessel is an important environmental factor that affects the water relations of cultured tissues (Jeong, Fujiwara and Kozai 1995). Relative

humidity is normally high in the culture vessel and may have some deleterious effects on cultured plantlets (see Fig.1.01). Several studies have demonstrated that lowering RH in the culture vessel improved the resistance of tissues to water loss (Wardle, Dobbs and Short 1983; Smith, Roberts and Mottley 1990; Smith *et al.* 1992).

Recently, there has been much interest in improving the aeration of plant tissues in cultures to overcome the adverse effects of ergastic gases. Adkins, Shiraishi and McComb (1989) pointed out that the inability of callus/tissue to grow well in culture has greatly hampered tissue culture research, and that to overcome the adverse effects of accumulated gases, culture vessels that would provide better gaseous exchange need to be developed. Debergh and Vanderschaeghe (1990) suggested that vitrification of *in vitro* grown plantlets may be overcome by improving ventilation and/or aeration in culture vessels. Rossetto, Dixon, and Bunn (1992) reported that by improving the diffusive aeration of *in vitro* grown rare Australian plants (species of *Conostylis*, *Diplolaena*, *Drummondita*, *Lechenaultia* and *Sowerbaea*), vitrification could be reduced, shoot quality improved significantly and the plants more easily acclimatized when transplanted into soil. They also suggested that diffusive aeration may be a simple and efficient method for improving a large number of plant species. 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).

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 Kubota 1995): growth of cuttings, seedlings and callus can all be improved. For example, Kozai, Kubota & Nakayama (1989) found higher photosynthetic rates and growth of strawberry 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 benefited 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

The main aim of the present study was explore the potential of a newly patented and simple system for force-ventilating plant tissue cultures with sterile humidified air, a system not requiring an electricity supply, pumps or compressed gases (Armstrong and Armstrong 1994a) The apparatus provides gas-flow by the processes of humidity-induced diffusion and convection: mechanisms which require only the establishment and maintenance of humidity differentials across a microporous partition (see Chapters II & III).

Humidity-induced diffusion was first recorded by Dufour (1874) He found that when a porous wall separates two air masses of differing humidity, then two opposed and unequal diffusion flows occur across the wall; he referred to this phenomenon as "diffusion hygrometrique". Kundt (1877) mentioned that "abundant flow moves from the drier air to the damper air" In particular circumstances the flow of dry air can result in a pressurisation of the damper air and cause a pressure flow - humidity-induced convection More recently humidity-induced convective flows have been found to occur in wetland plants Dacey (1981) described a pressurised flow-through ventilation system of gas transport in water-lilies (*Nuphar lutea*) and referred to the mechanism as Hygrometric Pressurisation Subsequently a number of papers have been published showing evidence of humidity-induced convective throughflows of gases in other species: *Nymphoides peltata* (Grosse and Mevi-Schutz 1987) and *Nelumbo nucifera* (Dacey 1987; Mevi-Schutz and Grosse 1988a,b), and substantial flows in the leaves of *Nymphaea alba* were recorded by Armstrong *et al* (1991) The first direct evidence of a convective throughflow of gases in a grass was reported for *Phragmites australis* (Cav) Trin. ex Steud. (Armstrong and Armstrong 1990). In this study they examined the effect of light on the convection in *Phragmites* and

demonstrated how humidity-induced throughflow convection can substantially increase rhizome and root aeration. It was also reported that in *Phragmites* this type of convection was particularly rapid under conditions of low atmospheric humidity and warm, sunny conditions (Armstrong and Armstrong 1991). Humidity-induced convection in *Phragmites* was mimicked using a physical model in which a Nuclepore membrane was used to imitate the leaf sheath stomatal surface of the plant (Armstrong 1992; Armstrong and Armstrong 1994b). This model, which was used to investigate the mechanism of the convective process (Armstrong, Armstrong & Beckett 1996a) forms the basis of the forced ventilation systems described and used in this thesis. The features which help induce the humidity-induced diffusion, also prevent the entry of fungal spores and bacteria and provide a humidified stream of air which has the potential to flush potentially toxic gases, e.g. ethylene from the culture vessels, and eliminate the need for the use of ethylene inhibitors or antagonists Such a system requires no pumps, compressed gas or electricity supply.

Two kinds of the new throughflow ventilation system have been used and these, and the theory underpinning them, are described in Chapter II together with other systems reported in the literature. Chapter III reports on the physical performance of the new apparatus, and the remainder of the thesis is concerned with their use in the culture of seedlings and micro-cuttings of several plant species: cauliflower (Chapter IV), tobacco (Chapter V), *Annona* (Chapter VII) and potato (Chapter VIII). Chapter VI is concerned with effects of the various ventilation treatments on leaf anatomy of tobacco and cauliflower plantlets.

Chapter IV describes the effects of the different types of ventilation system on the growth and development of cauliflower seedlings. The plants were cultured in the presence or absence of ethylene inhibitors and a precursor (ACC), and ethylene and $CO_2$ levels were monitored with the aim of establishing whether endogenous ethylene has a significant effect on growth and whether $CO_2$ supply with conventional capping systems can be limiting. The results of similar investigations on tobacco are reported in Chapter V. In Chapter VII a

system for the micropropagation of *Annona squamosa* and *Annona muricata* using nodal explants of green house grown plants is described. The effects of forced and diffusive ventilation on micropropagation, leaf and flower-bud abscission, growth and development, chlorophyll contents of the leaves and photosynthetic rate are examined. In Chapter VIII an *in vitro* tuberization technique for potato is described, and to improve the culture conditions a forced ventilation was introduced into the culture vessels by using the throughflow ventilation. Finally, Chapter IX summarises the main findings of the project, indicating that there are obvious beneficial effects to be gained from the use of the new and simple ventilating system.

# CHAPTER - II

## THE STRUCTURE AND THE MECHANISM OF THE VENTILATION SYSTEM

### 2.1. INTRODUCTION

Forced ventilation of the culture vessel head-space in plant tissue culture or micropropagation is a comparatively recent development. This chapter reviews various methods which have been used to achieve head-space ventilation, describes in detail a new type of apparatus for generating flows, and discusses the mechanism and mathematics underlying its operation. Its physical behaviour is further explored in Chapter III and the remainder of the thesis is concerned with studies of its application to plant micropropagation.

The new apparatus has been built in two forms and is covered by British Patent No. 9302932.0 (Armstrong & Armstrong 1994a). In its original form, (called here System I), it was first described in the literature by Armstrong *et al.* (1996) and has separate inflow and outflow turrets with polycarbonate Nuclepore membranes (pore diameter 0.03 µm and 0.2 µm respectively). The inflow membrane provides the porous partition to achieve pressurised throughflow by humidity-induced diffusion, while comparatively free venting is allowed through the outflow membrane (see below). The major disadvantage of this system is the need to adjust daily the water level of the inflow turret to keep it as near to the membrane as possible. Another drawback is that the turrets are connected to the culture vessel by means of flexible tubing which makes it necessary to use two separate clamps to hold the inflow and the outflow turret. The second, more compact system, (System II), was designed specially for the current study and combines the inflow and outflow parts in a single unit. This model requires less regular attention. With both types of model the membrane pore sizes are sufficiently small to exclude bacterial and fungal spore contamination and the forced ventilation produced by the apparatus is in the form of a sustained stream of sterile

humidified air at flow rates of 1-2 $cm^3$ $min^{-1}$ (with inflow membrane diameter = 25 mm), or about 5.0 - 9.0 $cm^3$ $min^{-1}$(with inflow membrane diameter = 50 mm)

## 2.2. HISTORICAL BACKGROUND

Plant tissue culture has a long history, dating back to the work of Gottlieb Haberlandt and others at the end of the 19th century, but the concepts and techniques of forced ventilation developed for plant tissue culture are less then a decade old. Probably the first system developed to give a favourable *ex vitro* environment of cultures grown *in vitro* was demonstrated by Kozai *et al.* (1987); this was later developed by Hayashi and Kozai (1987) (Fig. 2 01) Although this computer-controlled unit was only for the improvement of plantlets during the acclimatization period, it was able to control temperature, humidity, light intensity, $CO_2$ concentration, air velocity and nutrient solution temperature of the culture vessels

In the following year (1988) Shimada, Tanaka and Kozai described another ventilation system to estimate net photosynthetic rates of C-3 plants (*Primula malacoides*) cultured *in vitro* (Fig. 2.02). In the same year Fujiwara, Kozai and Watanabe developed a forced $CO_2$ enrichment technique for plant tissue culture (Fig 2 03) using a specially equipped growth chamber. The chamber contains a $CO_2$ control unit consisting of a container with pure liquid $CO_2$, an electric solenoid valve with a relay for opening and closing the solenoid, and an infrared type $CO_2$ controller with an air pump for air sampling. Two identical transparent acrylic boxes containing culture vessels were placed in the chamber. Air mixing was operated by a microfan in each box to provide an even distribution of $CO_2$ concentration. Air exchange between the inside and the outside of the growth chamber was provided to a certain degree to maintain the $CO_2$ concentration in the chamber at more or less the same level as the atmospheric $CO_2$ concentration (350 ppm).

In 1989 another apparatus was reported by Walker, Heuser and Heinemann to determine the effect of ventilation on Stage II micropropagation of *Rhododendron* 'P.J M.'.

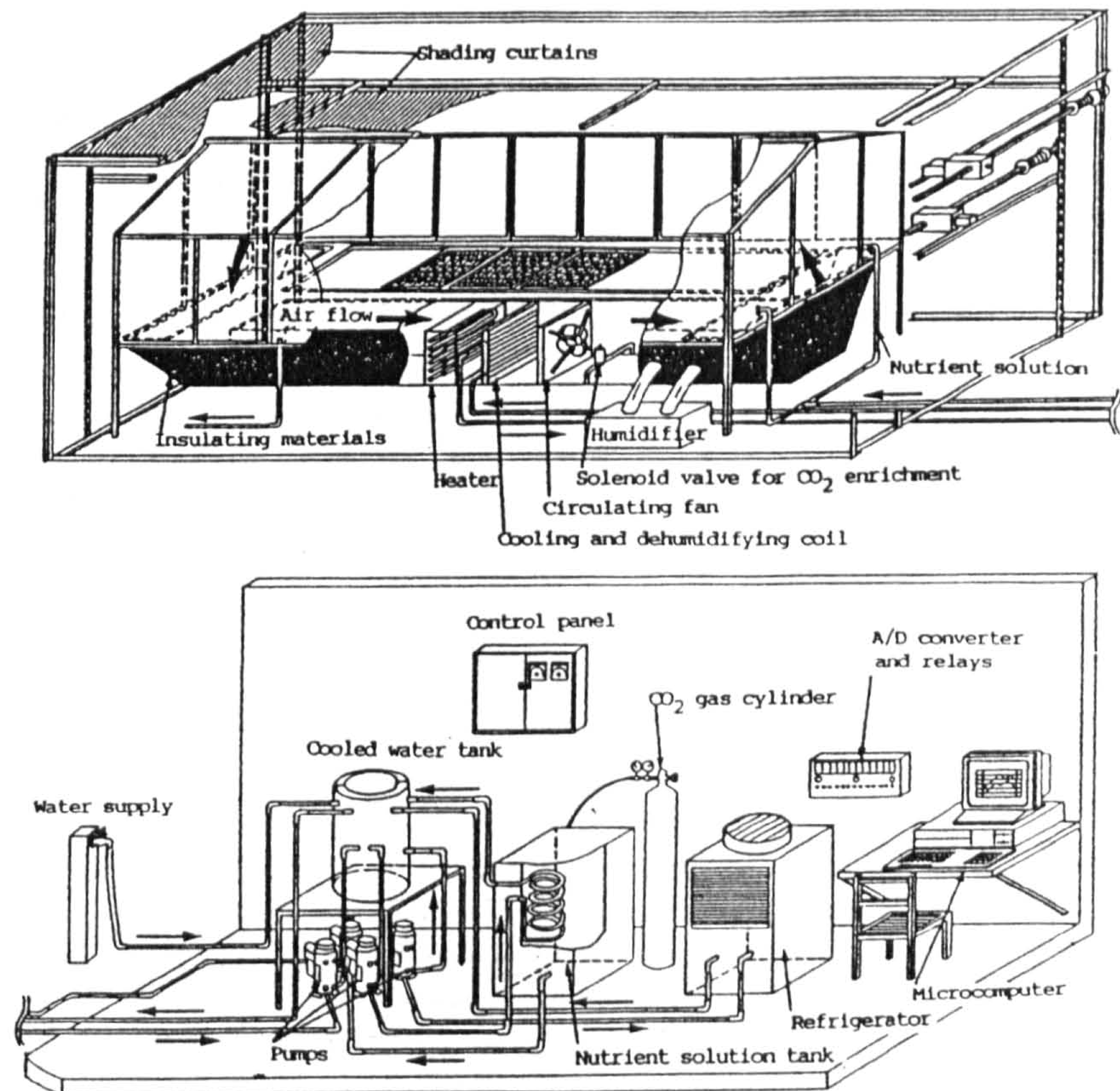



**Fig. 2.01.** Schematic diagram of an acclimatization unit for increasing the percent survival *ex vitro* of plantlets cultured *in vitro* and for accelerating the subsequent growth *ex vitro* (Hayashi and Kozai 1987).

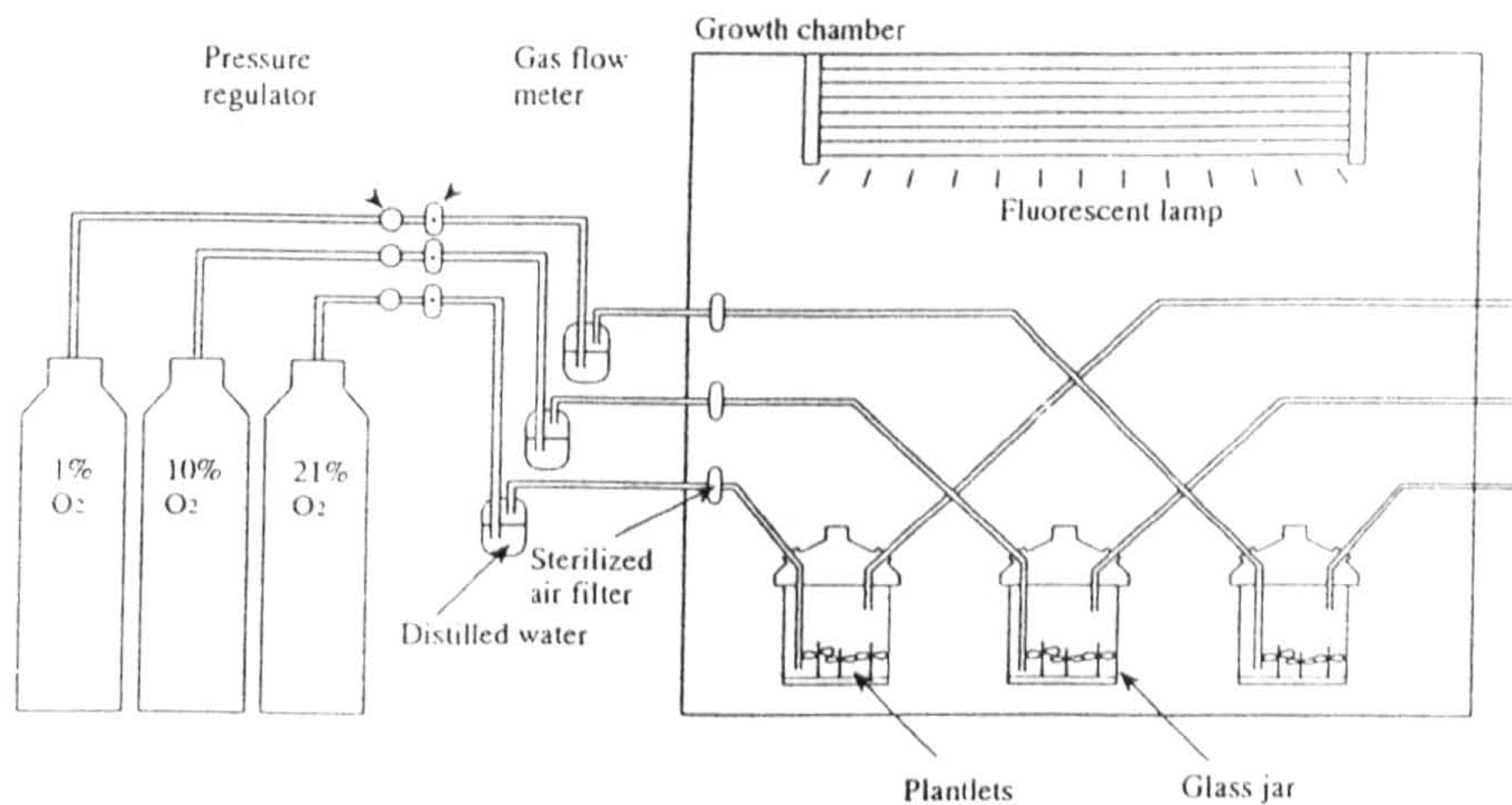



**Fig. 2.02.** Schematic diagram of an apparatus capable of estimating the net photosynthetic rates of plantlets *in vitro* at 3 different $O_2$ concentrations. The moistened gas with 366 μmol $mol^{-1}$ $CO_2$ and 3 different $O_2$ concentrations (the rest being nitrogen gas) is passed across the glass jar (culture vessel) at a flow rate of 1-4 ml $s^{-1}$ (Shimada, Tanaka and Kozai 1988).

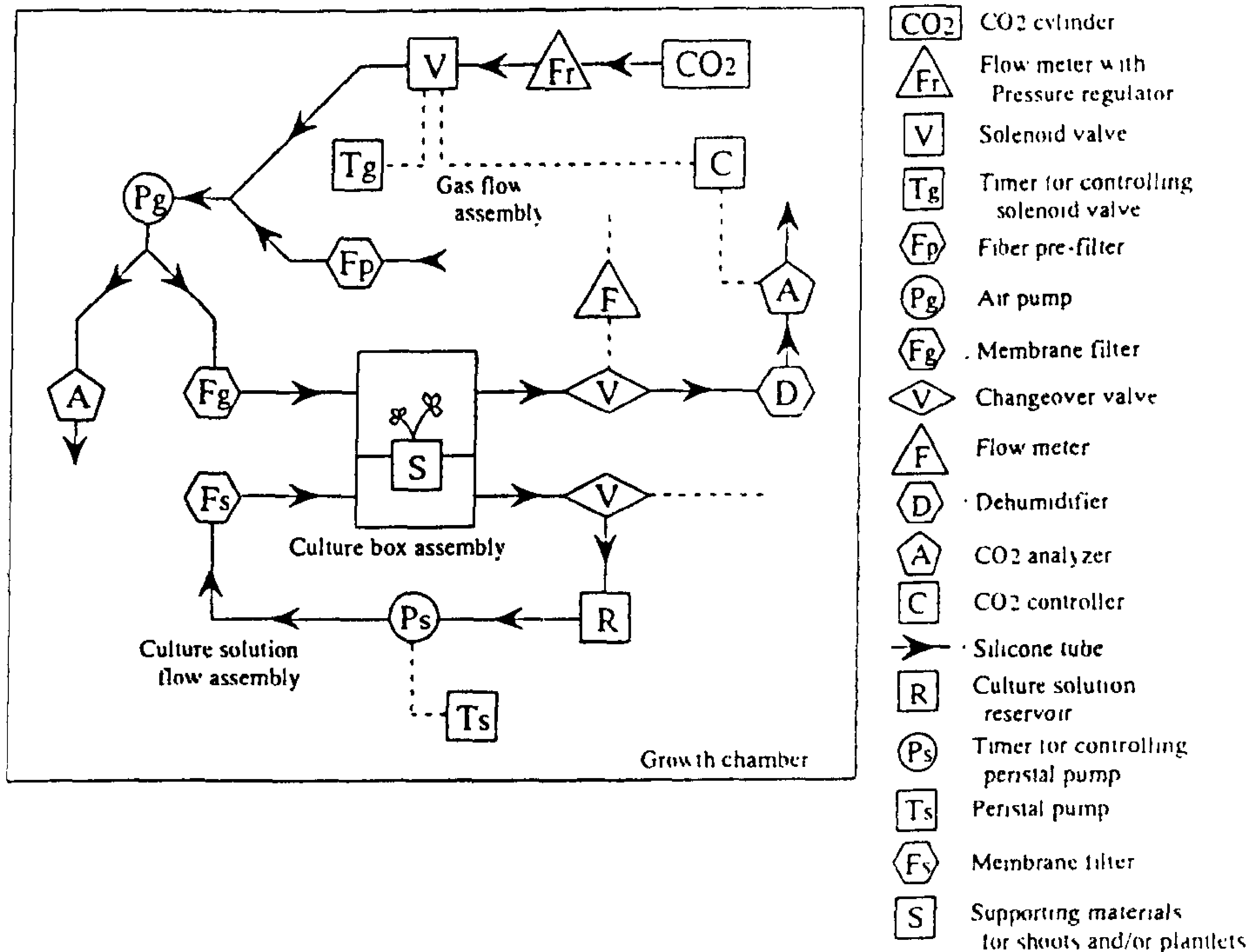



**Fig. 2.03.** Schematic diagram showing a configuration of photoautotrophic tissue culture system (Fujiwara, Kozai and Watanabe 1988). Gas and culture solution flow lines are represented by solid lines and electrical signal lines by dashed lines.

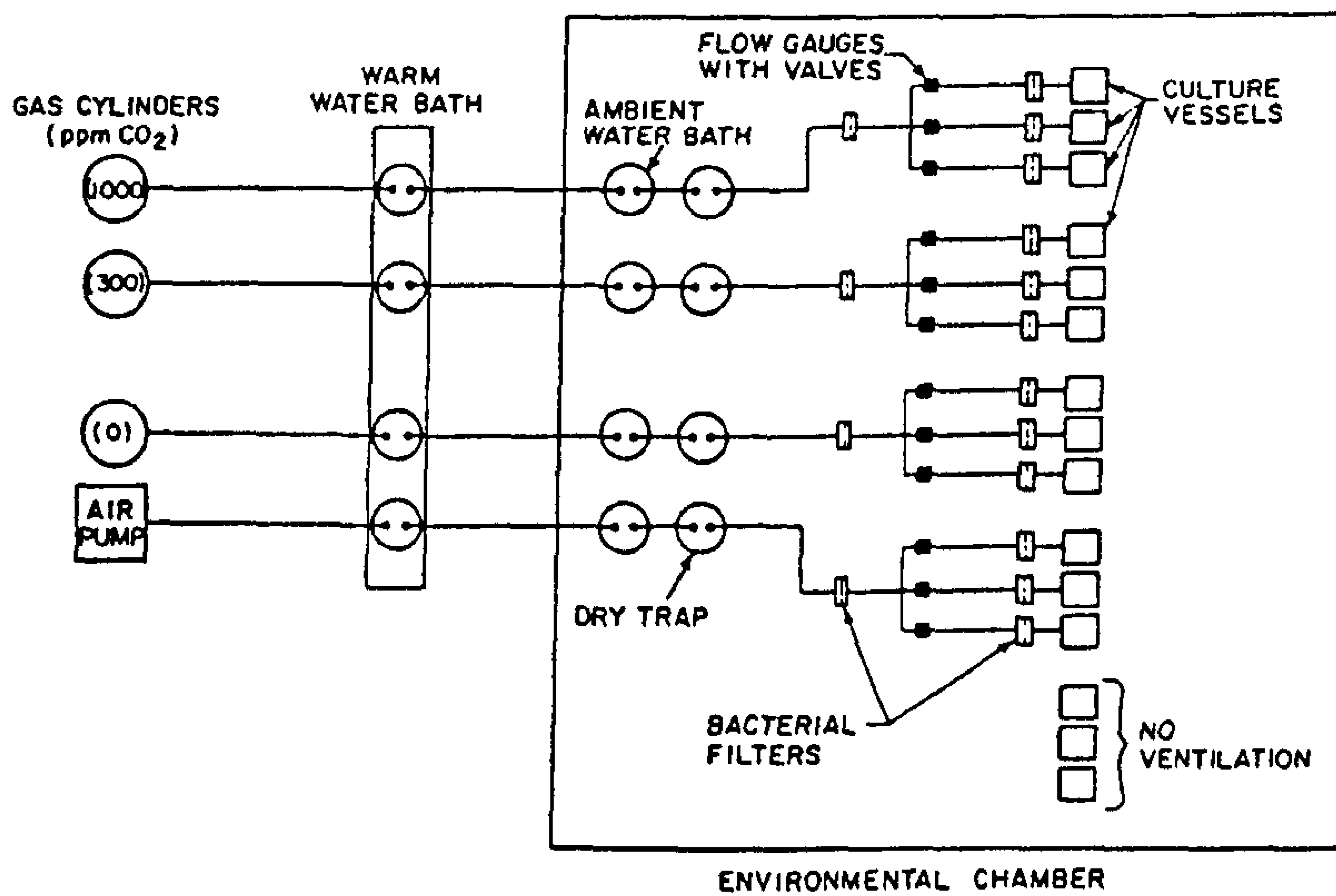



**Fig. 2.04** Schematic diagram of the experimental apparatus developed by Walker, Heuser and Heinemann (1989) to determine the effects of forced ventilation on stage II micropropagation of *Rhododendron* 'P.J.M.'.

To provide 0, 300 and 1000 ppm carbon dioxide gas treatments, gas mixtures were provided from different gas cylinders (Fig. 2.04), and the atmospheric air treatment was supplied by a diaphragm-type air pump. The ventilation gas for each treatment was bubbled through two water baths to saturate it with water vapour and prevent dehydration of the agar and cultures.

At the same time (1989) Adkins, Shiraishi and McComb developed a continuous gas-flow system for the study of callus growth. The system allows for several gases to be mixed and passed through culture tubes containing callus on Miracloth boats placed on a filter paper bridge as shown in Fig. 2.05. Exiting gases can be monitored for ethylene (and other hydrocarbons), ethanol (and acetaldehyde), $O_2$ and $CO_2$.

To generate low (30-65%), medium (70-95%) and high (97% and above) relative humidities in the culture vessels Kozai *et al.* (1990) developed a forced ventilation system (Fig. 2.06). In this system a desiccant (silica gel) contained in a flask was used to dehumidify the air which was blown through it by an air pump. The major draw back of this system is the desiccant which must be replaced with new desiccant or re-dried after a certain time interval to maintain its water absorbing capacity.

In the following year (1991) Nakayama, Kozai and Watanabe set up an experiment for measuring $CO_2$ exchange rates or net photosynthetic rates of cultures under forced ventilation (Fig. 2.07). Oxygen, $CO_2 + N_2$, and $N_2$ gases from separate containers are mixed and then humidified with distilled water and finally introduced into the vessel at predetermined flow rates and predetermined $CO_2$ and $O_2$ concentrations. The $CO_2$ and $O_2$ concentrations can be changed by changing the mixing ratio of the $O_2$, $(CO_2 + N_2)$ and $N_2$.

In the same year (1991) Fujiwara, Ota and Kozai developed a device to estimate simultaneously transpiration and photosynthetic rates of cultures under forced ventilation (Fig. 2.08). A feature of this device is that it is possible to measure the temperature and relative humidity of incoming and outgoing air to/from the culture vessels with temperature and the relative humidity sensors. Another similar type of experiment was set up by the same

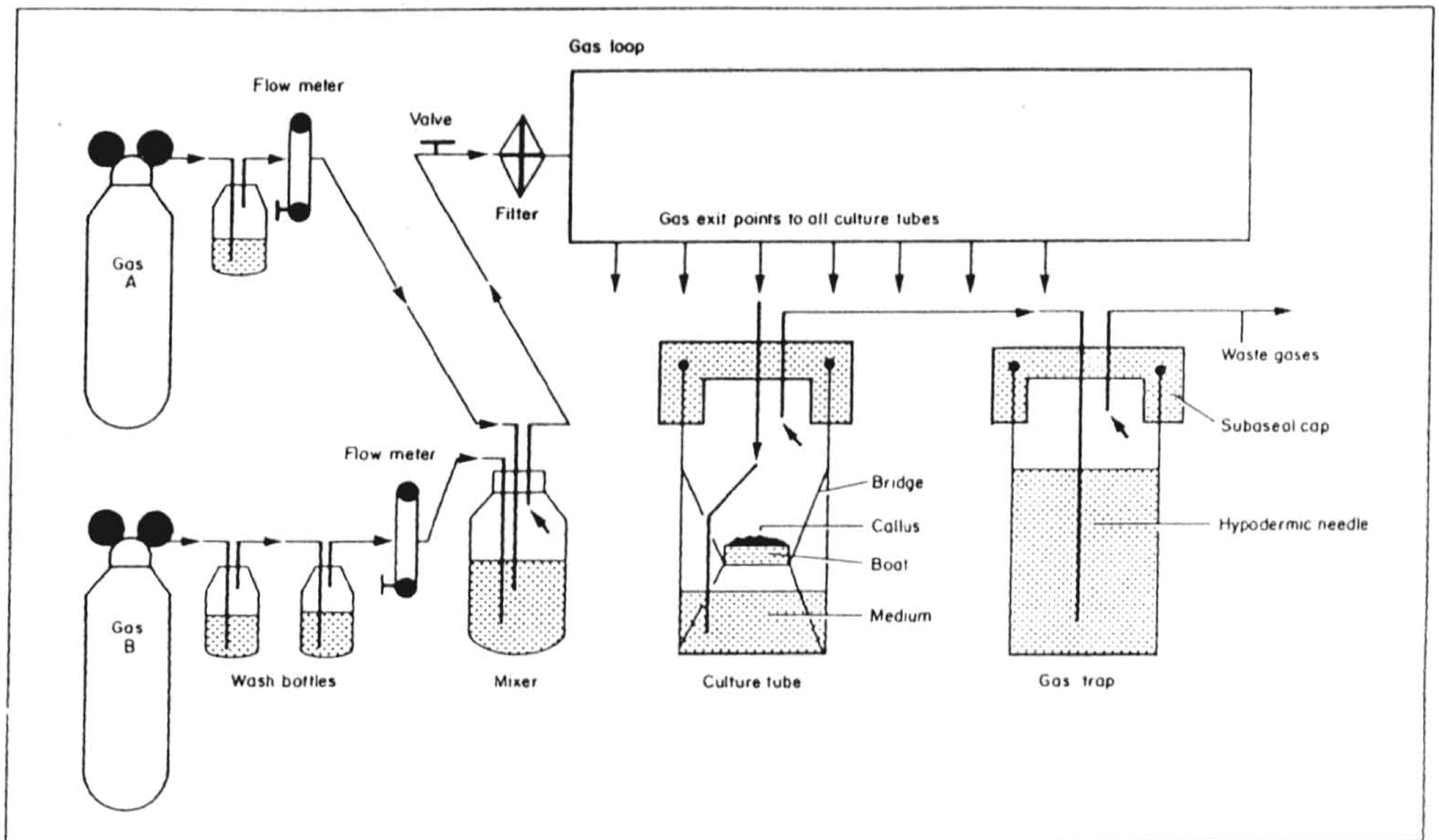



**Fig. 2.05.** The gas-flow system used in the constant environment study. The tests are carried out in culture tubes attached to a gas loop housed in a dark constant-temperature room (30±2°C). Callus is growing on a Miracloth boat and placed on a filter paper bridge soaked in liquid medium. Features of the gas-flow system include 1) wash bottles to scrub incoming gases, 2) flow meters to mix gases, 3) filters to keep cultures sterile, 4) traps to collect and concentrate volatiles and 5) injection valves to sample for $O_2$ and $CO_2$ (Adkins, Shiraishi and McComb 1989).

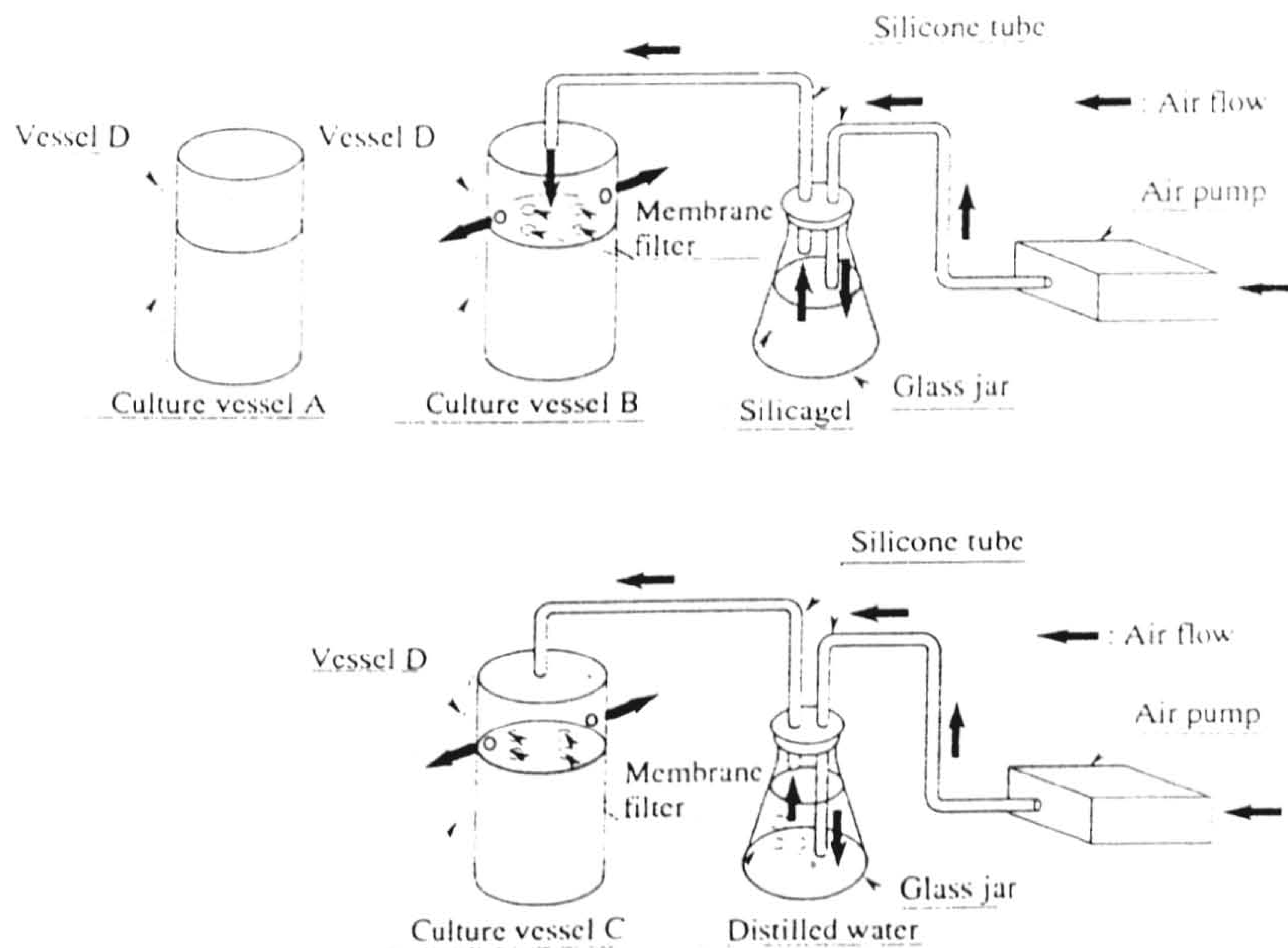



**Fig. 2.06.** Schematic diagram showing a simple method for generating low, medium and high relative humidities in culture vessels (Kozai *et al.* 1990). Treatment A (culture vessel A) : high relative humidity (97% and above), ). Treatment B (culture vessel B) : low relative humidity (30-65%), treatment C (culture vessel C) : medium relative humidity (70-95%).

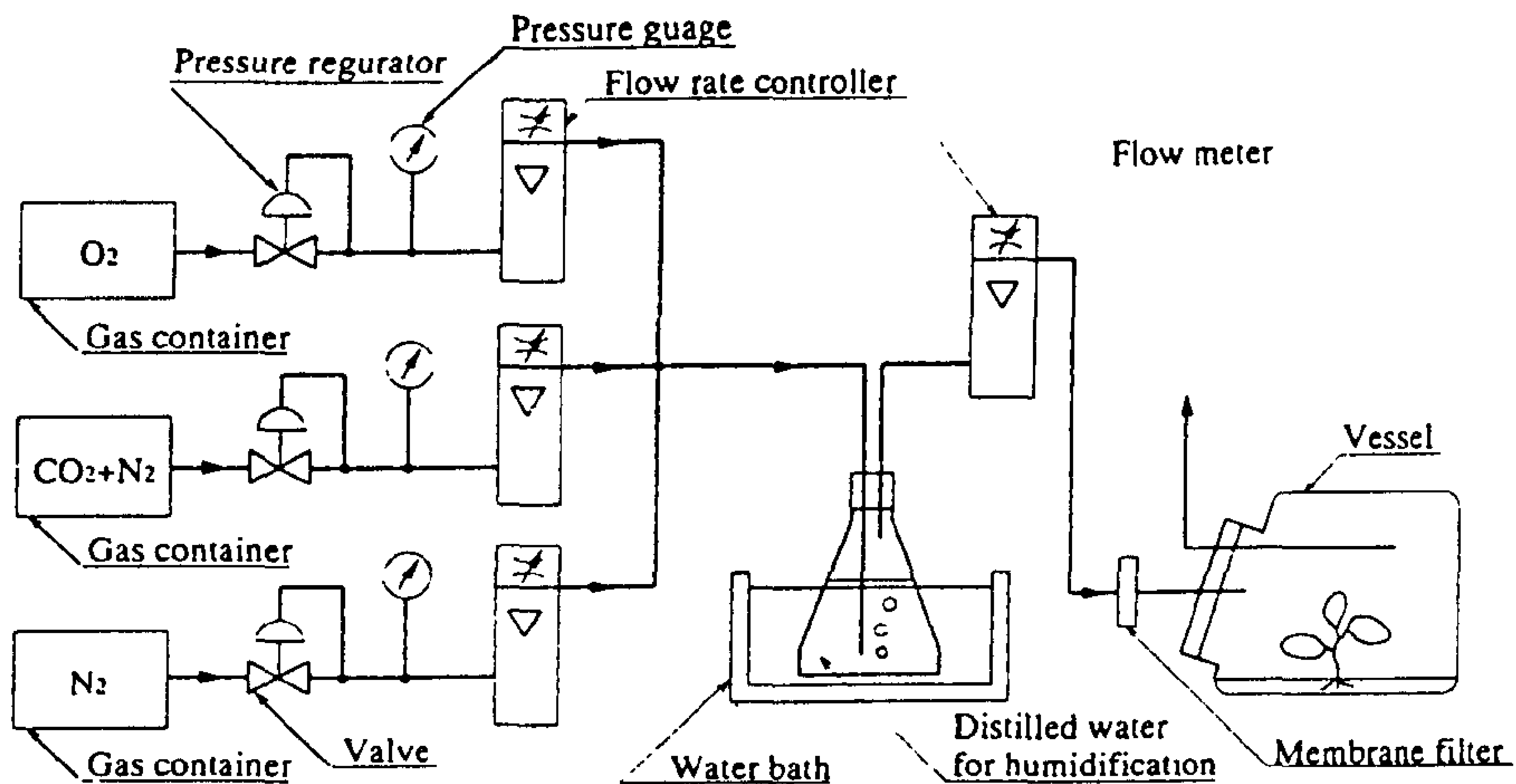



**Fig. 2.07.** Schematic diagram of a system for estimating net photosynthetic rates of explants/shoots/plantlets *in vitro* under forced ventilation conditions (Nakayama, Kozai and Watanabe 1991).

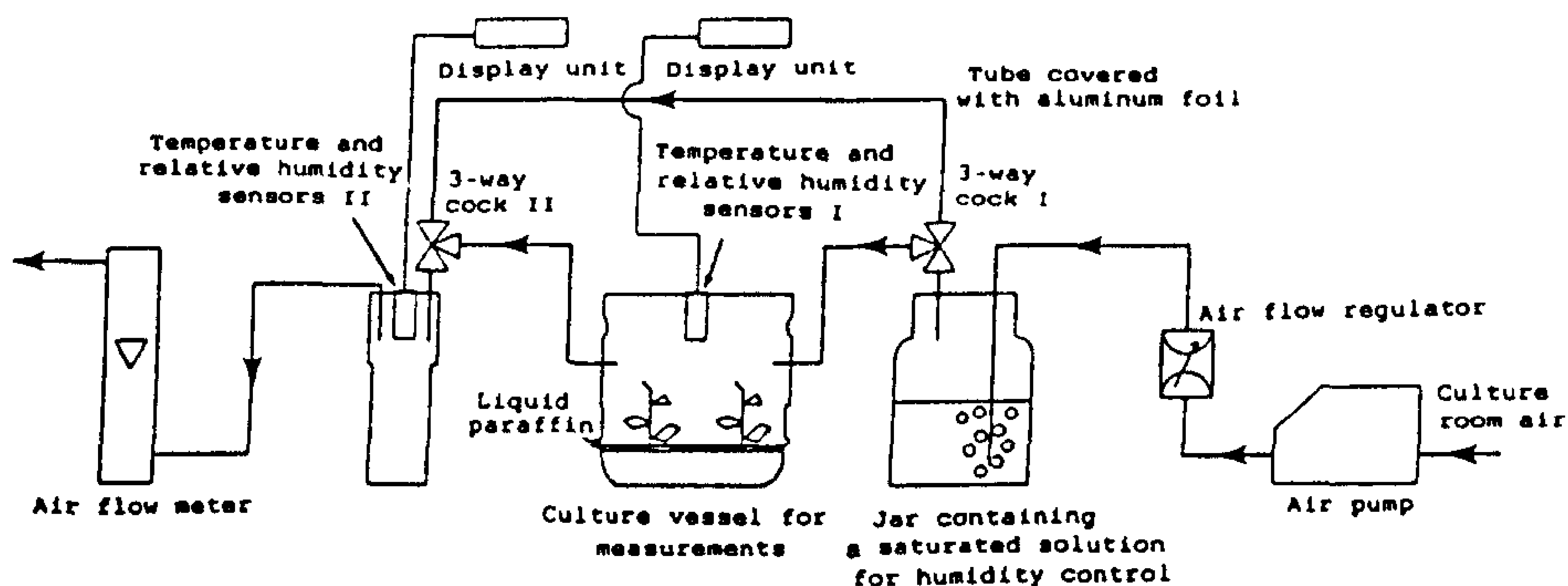



**Fig. 2.08.** Schematic diagram of a system for estimating transpiration and net photosynthetic rates of *in vitro* plantlets at different relative humidities (Fujiwara, Ota and Kozai 1991). Temperature and relative humidities of incoming and outgoing air into/from the culture vessel are measured with temperature and relative humidity sensors II by turning 3-way cocks I and II properly. A quarter milliliter of air is sampled at points A and B with a gas-tight syringe for measurements of $CO_2$ concentration of incoming and outgoing air with a gas chromatograph.

authors (Fujiwara, Kira and Kozai 1992) to measure concurrently net photosynthetic rates for three different culture (vessel) conditions (e.g. medium composition, plant species, growth stage etc.). As shown in Fig. 2.09 diluted $CO_2$ gas with $N_2$ can be further diluted by air, and the $CO_2$ concentration of the incoming air was lowered by a $CO_2$ absorbing agent before being sent to the culture vessels. A similar system has been developed for measuring the $CO_2$ exchange rate of *in vitro* plantlets by Ebert, Karstens and Ludders (1993) (figure not shown).

Fujiwara *et al.* developed another device in 1993 for experiments on the physical environmental effects on growth and development of cultures (Fig. 2.10a). This device was 70 cm wide, 45 cm deep and 70 cm high The upper part of this device consists of a light source and a culture box containing culture vessels The lower part is the control box with a control panel (Fig 2.10b). The $CO_2$ is maintained at a certain level by adjusting the flow rates of pure $CO_2$ from the container (volume . 450 ml) and/or incoming air.

Kitaya and Sakami (1993) made a system for $CO_2$ enrichment of chlorophyllous callus by utilizing the respiratory $CO_2$ produced by a crop of mushrooms. As shown in Fig. 2.11 a plant tissue culture box was connected to a mushroom culture box using a semi-closed piping (silicone tube) system attached with ethylene absorbent, air pump, solenoid valve, etc. This system was designed as a prototype to use in space farming in the 21st Century (Kozai *et al.* 1995) but like others it is rather complex and requires electricity, microcomputer and sophisticated valve and air pumps. However one important feature was that, unlike others, the source of $CO_2$ was free of cost and did not require any gas cylinder.

To control relative humidities in the culture vessels Fujiwara, Aitken-Christie and Kozai (1993) developed another system where RH's of the culture boxes were maintained to control the vessels' RH (Fig. 2.12). This was achieved by connecting the box through an inlet pipe to either distilled water or saturated salt solutions in large Erlenmeyer flasks.

In the same year Yue, Gosselin and Desjardins (1993) developed a forced ventilation system (Fig. 2.13) in which the RH of the culture vessels could be controlled by adjusting

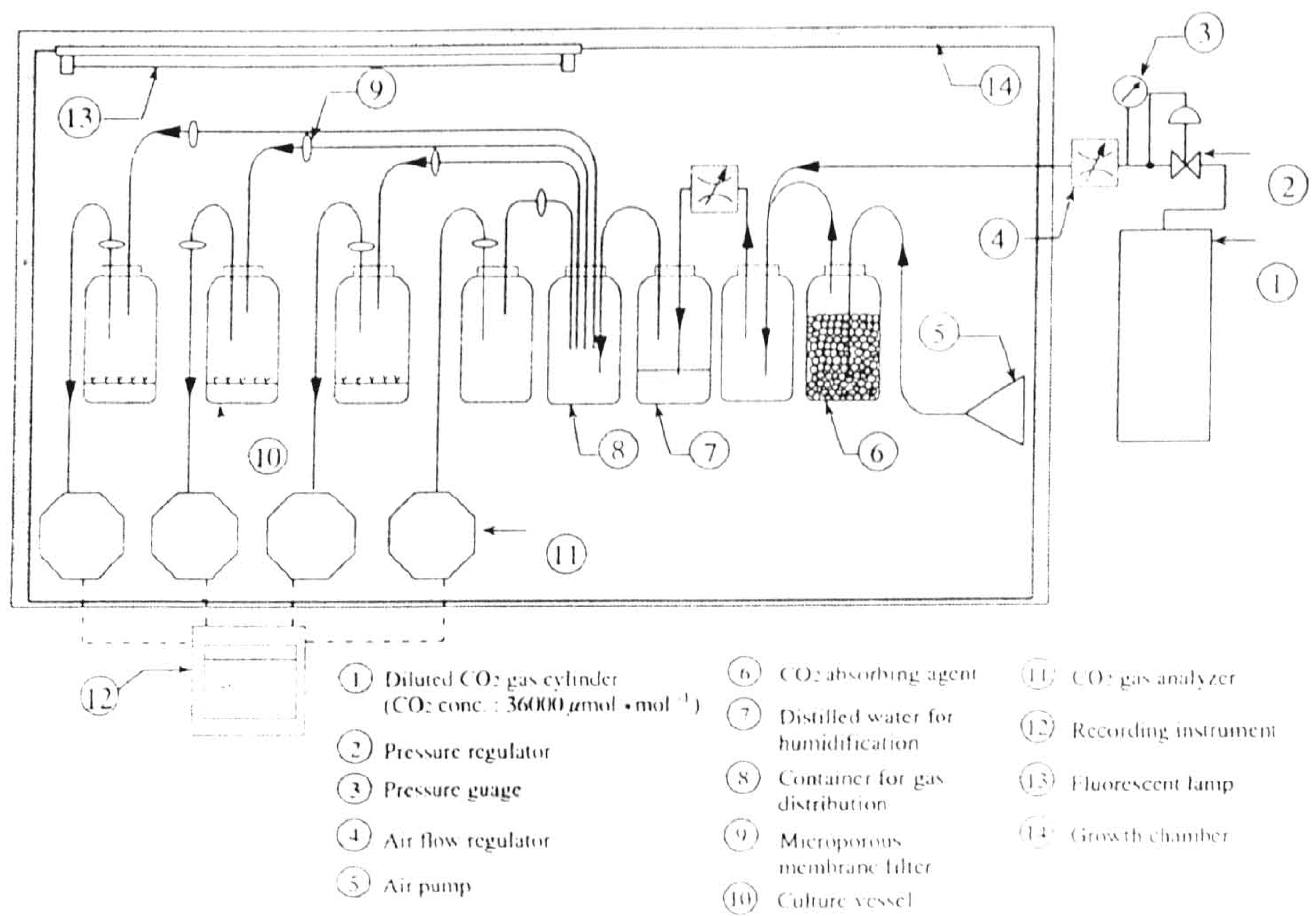
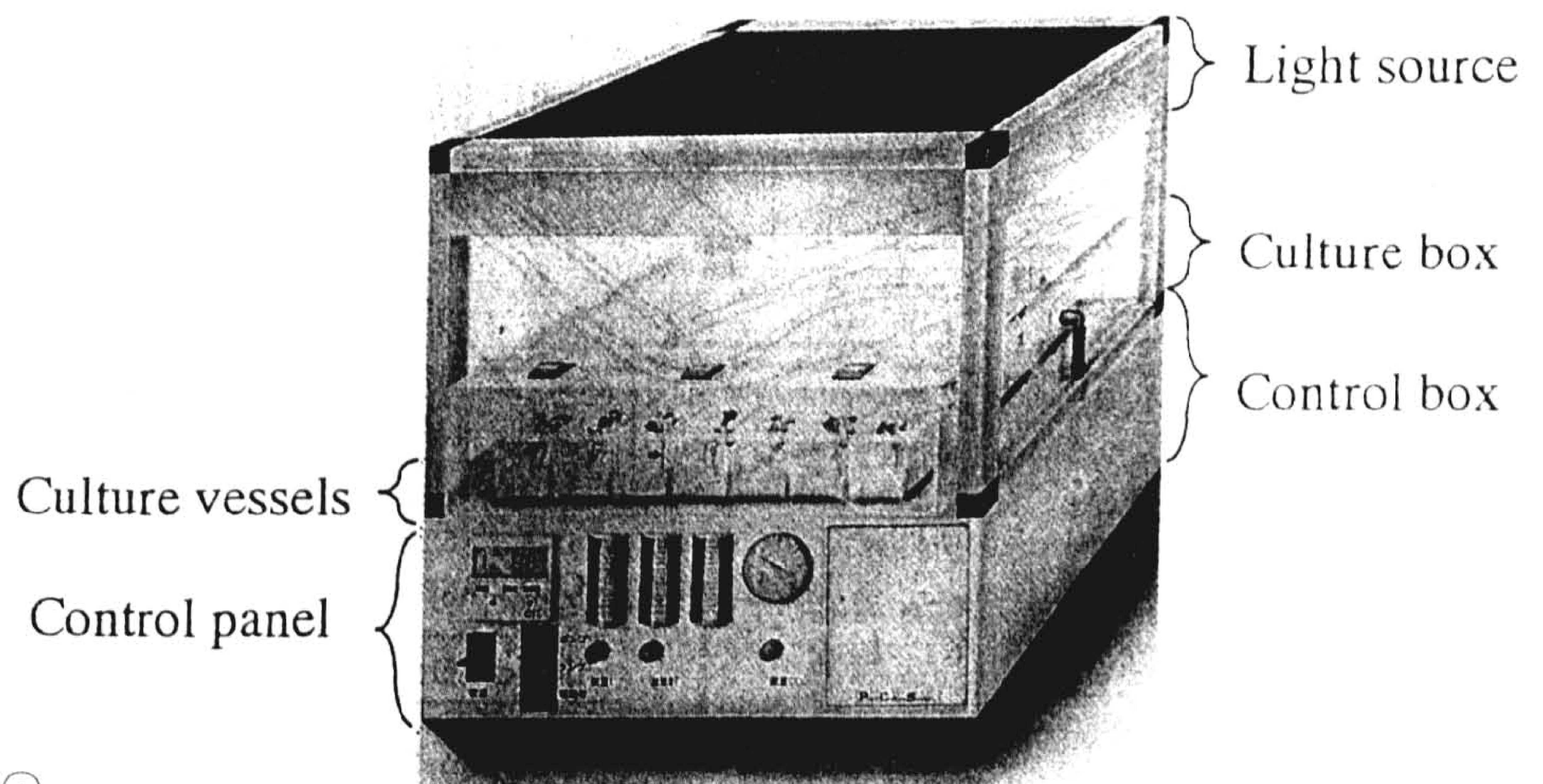



**Fig. 2.09.** Schematic diagram of an experimental setup for measuring $CO_2$ concentrations of inflow and outflow air for the culture vessels to estimate time courses of $CO_2$ exchange rates of plantlets *in vitro* (Fujiwara, Kira and Kozai 1992).



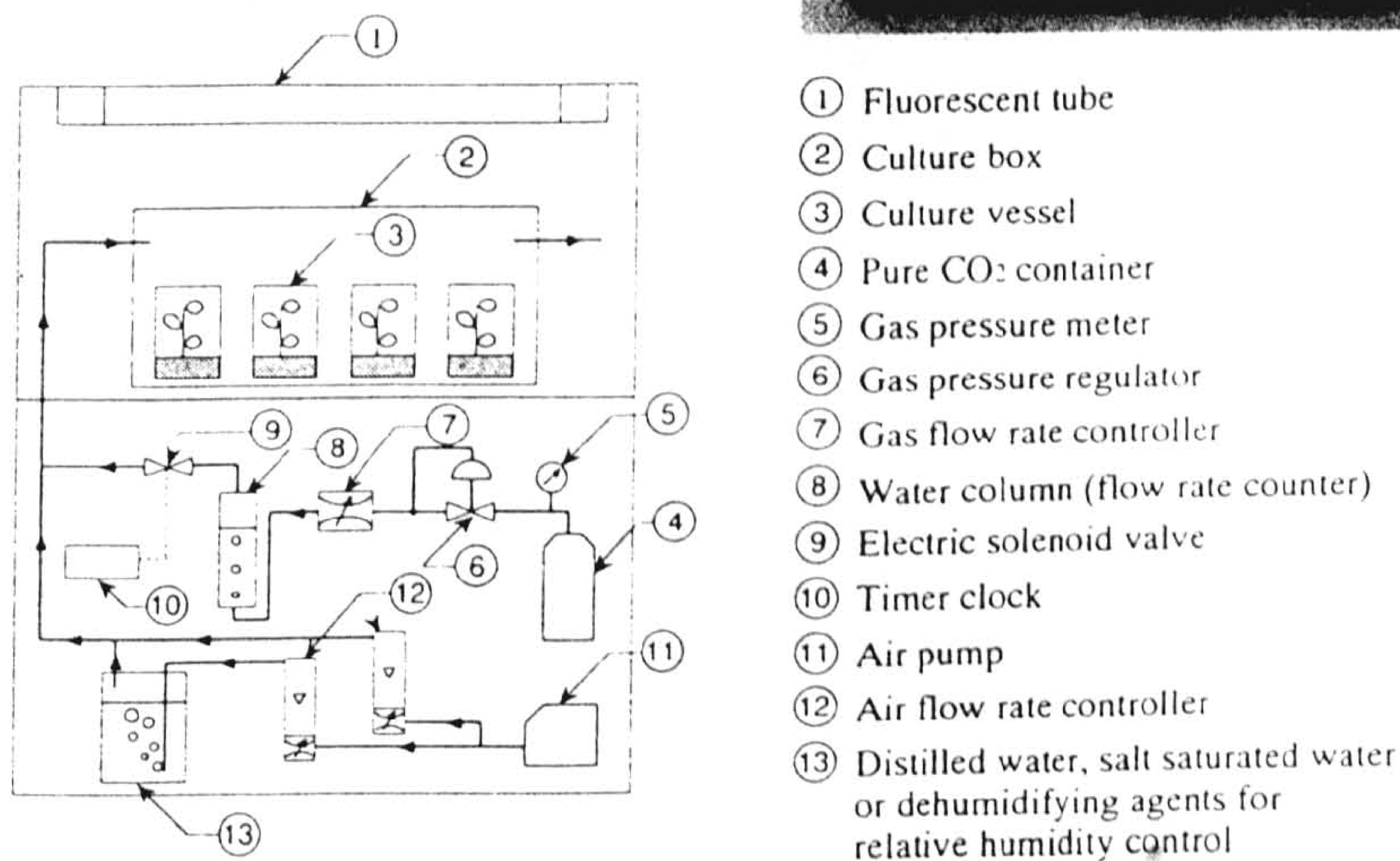




**Fig. 2.10.** a) Photograph showing of a simple device for $CO_2$ enrichment experiments under different lighting and humidity conditions b) schematic diagram of (a) showing the configuration and components of the system (Fujiwara *et al*, 1993).

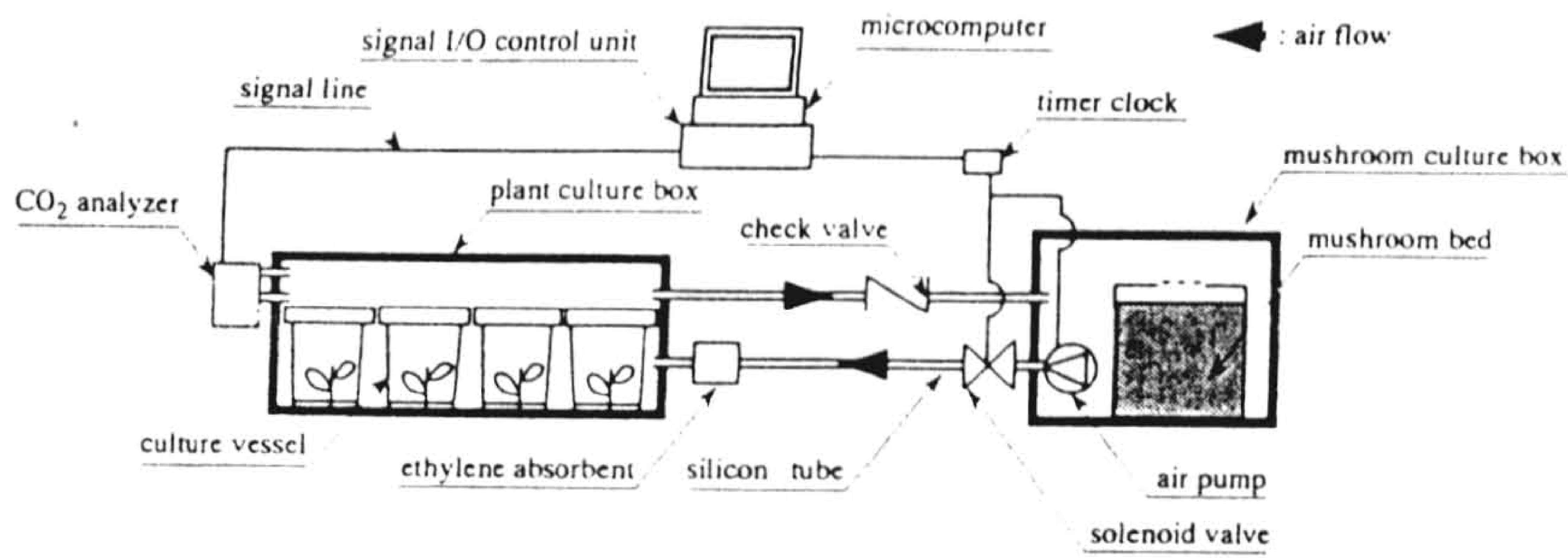



**Fig. 2.11.** Schematic diagram of a $CO_2$ enrichment system for plantlets *in vitro* using $CO_2$ produced by mushroom (Kitaya and Sakami 1993).

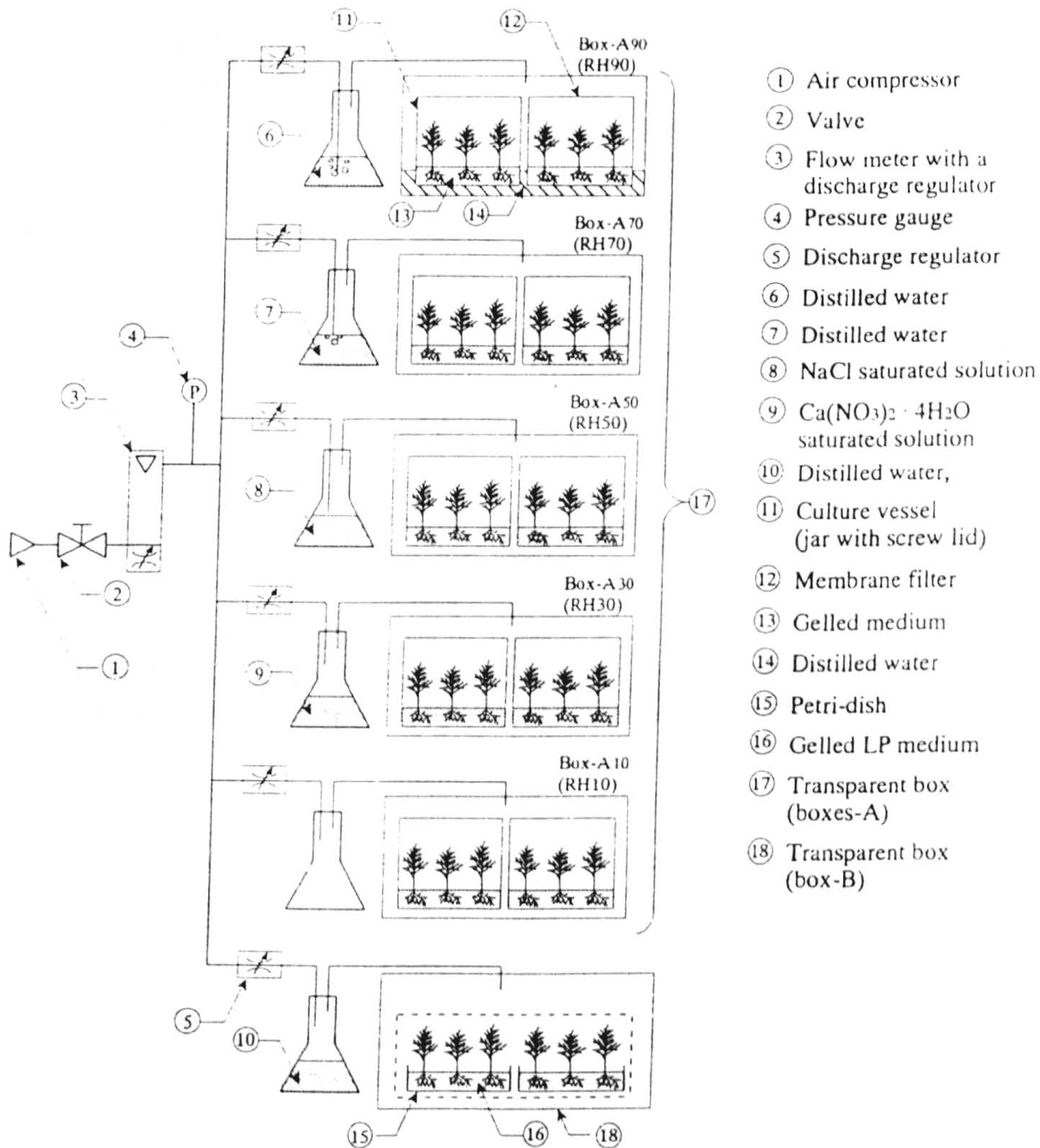



**Fig. 2.12.** Schematic diagram of a device for controlling humidities, using salt-saturated solution or distilled water, in transparent boxes (boxes-A and box-B) containing culture vessels with shoots (Fujiwara, Aitken-Christie and Kozai 1993).

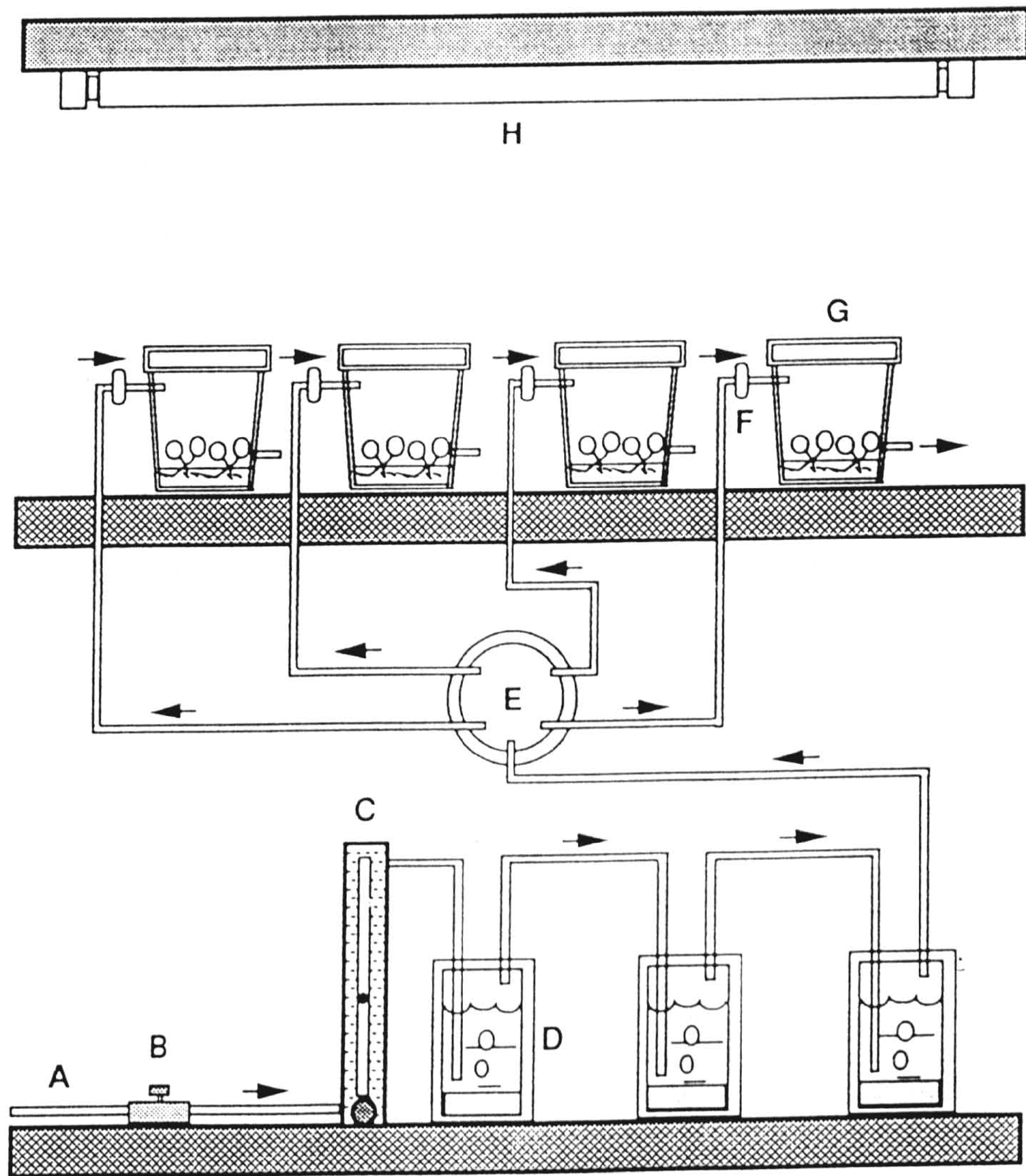



**Fig. 2.13.** Schematic view of an apparatus used to ventilate culture vessels with air of different relative humidities. Only one unit for one level of relative humidity is shown in this figure. Air from outdoors (A) entered through an air-flow controller (B) and a variable-air flowmeter (C) and was sent to three bubblers (D) to adjust the humidity. Depending on the relative humidity required for each treatment, bubblers contained distilled water or a saturated solution of KCl, NaCl or $K_2CO_3$. Actual relative humidities were approximately 100, 91, 78 and 46% at a temperature of 23°C. Air was then sent to a container (E) and delivered to four culture vessels (G, 6 X 6 X 9.5 cm) through membranes filters (F, 0.2 µm). In this experiment, air flow through each culture vessel was 100 mL $min^{-1}$) (Yue, Gosselin and Desjardins 1993).

the RH of inlet outdoor air (385 - 420 ml $l^{-1}$ $CO_2$) to each of four constant levels, i.e., 100, 91, 78 and 46%.

However, the mutifarious systems described here to achieve forced ventilation have not been successful enough to be used widely This is probably due in no small measure to their complexity and mechanisation requiring compressed gases or electricity-driven pumps, filtration systems, and often gas-mixing/material devices to maintain the flow balance to the culture vessels. The forced ventilation systems used in the current study and described below are much simpler. However, as will be shown in subsequent chapters, they have proved to be very effective.

**2.3. THE HUMIDITY-INDUCED DIFFUSION BASED VENTILATING SYSTEMS**

The two systems described are .

a) System I (Fig 2.14, Plate 2.01) having separate inflow and outflow turrets, and

b) System II (Fig 2.15, Plates 2.02, 2.03) having the inflow and outflow turret membranes in the same unit.

**2.2.1. System I :**

Polycarbonate Nuclepore membranes of known pore size (diameter 25 mm, thickness 10 μm and porosity 10%) are attached by silicone rubber compound across the 'mouths' of specially made cylindrical glass turrets (internal diameter 20 mm; outer diameter 24 mm; length 26 mm) which forms the inflow and the outflow parts of the ventilation system (as shown in Fig. 2.14). One inflow and one outflow turret is each connected by PVC tubing (ID = 4.0 mm; OD = 5.5 mm) to each tissue culture vessel (length = 77 mm; ID = 32 mm, total capacity = 60 $cm^3$).

The inflow turret is topped by the smaller-pored membrane (membrane pore diameter, MPD = 0.03 μm) and has a side tube for filling the turret with sterile water to maintain the water level close to, but not touching the membrane. A glass tube (OD = 6.75

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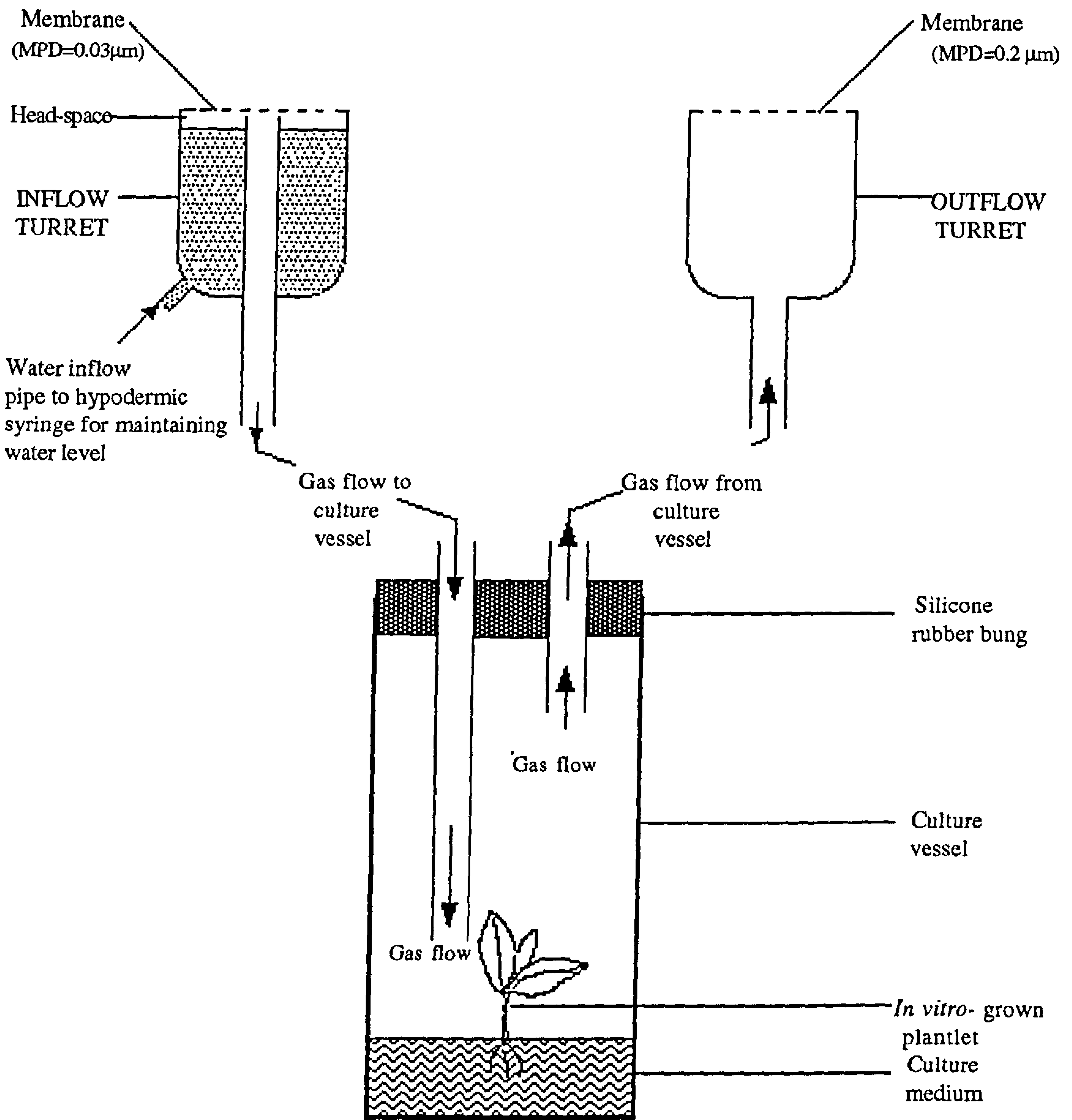
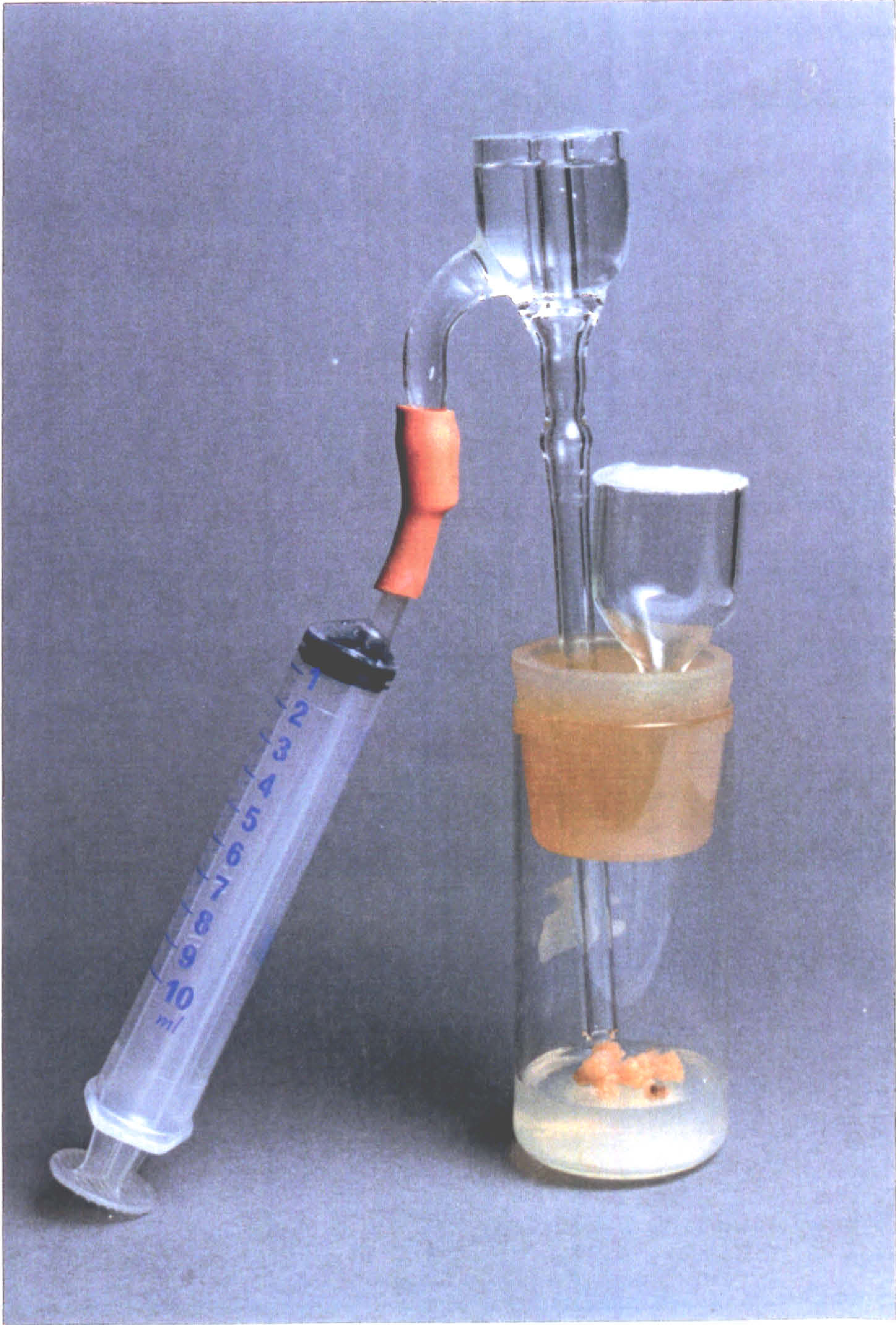



**Fig. 2.14.** Schematic diagram of the ventilation apparatus (System I) for plant tissue culture.

**PLATE : 2.01**

Forced ventilation apparatus (System I) for plant tissue culture; flow rate 1.0 - 1.5 $cm^3 min^{-1}$). Inflow turret (upper) containing water with syringe for refilling; outflow turret (lower) dry. Inflow membrane pore diameter = 0.03 μm; outflow membrane pore diameter = 0.2 μm; both membrane diameters = 25 mm. (X1.2). *cf.* Fig. 2.14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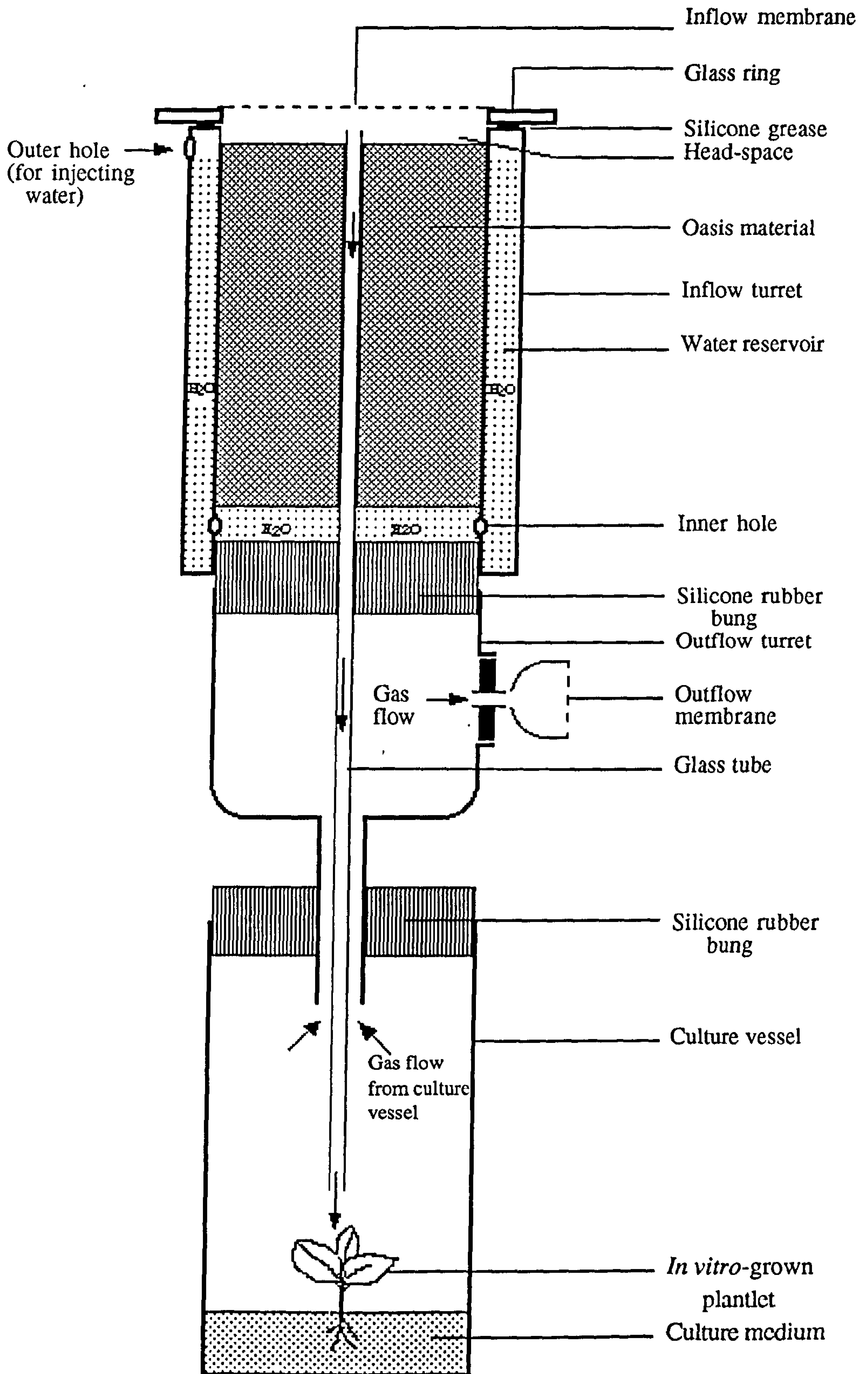



**Fig. 2.15.** Schematic diagram of the ventilation apparatus (System II) for plant tissue culture. Pore diameter: inflow membrane = 0.03 - 0.05 μm; outflow membrane = 0.2 μm.

**PLATE : 2.02**

Forced ventilation apparatus (System II) for plant tissue culture. Inflow turret (upper) containing green Oasis material surrounded by water reservoir; outflow turret (lower) with outflow membrane to the side. *cf.* Plate 2.03 and Fig. 2.15.

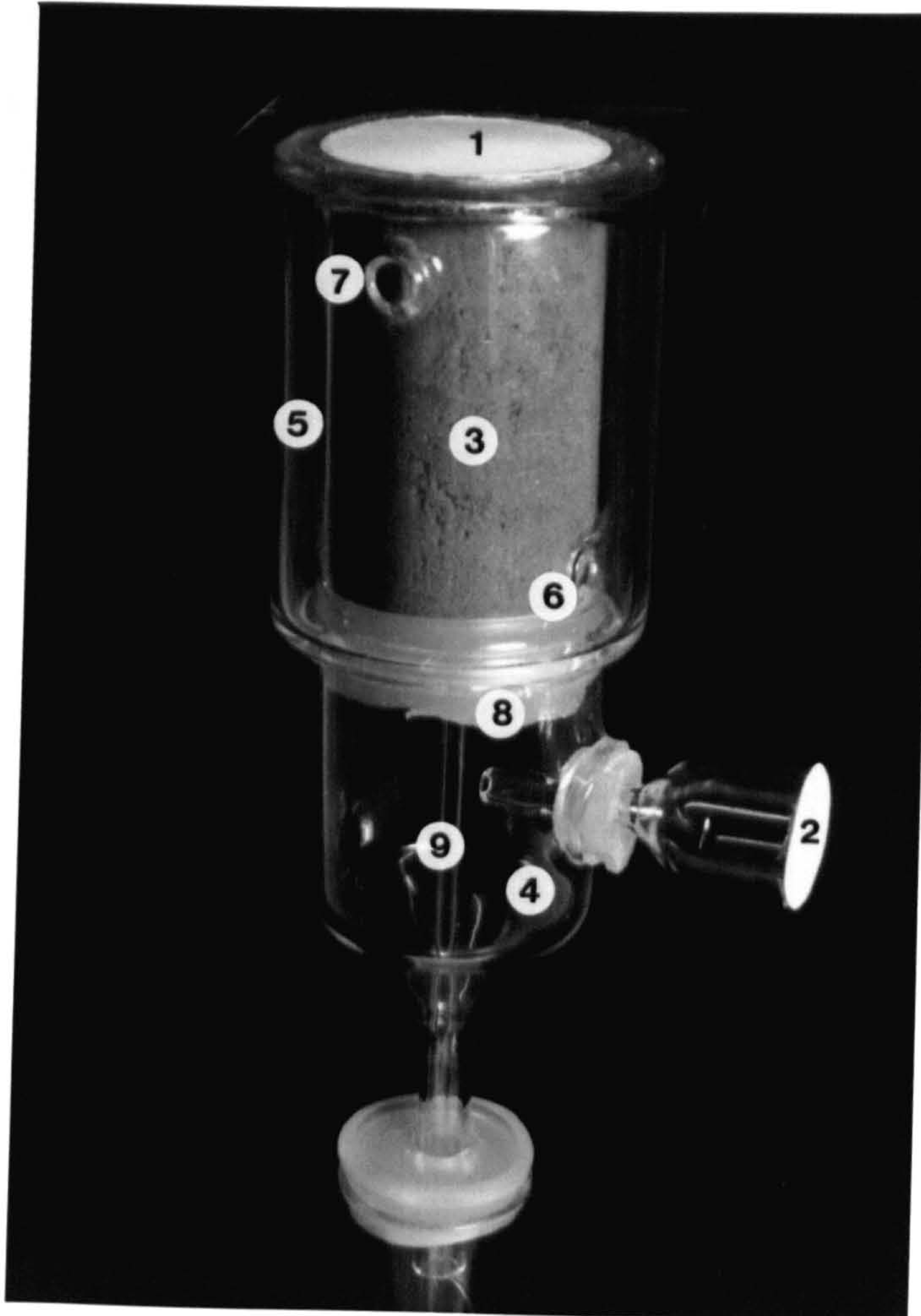
Left hand side : System IIF (fast flow apparatus; flow rate 3.5 - 5.0 $cm^3 min^{-1}$ inflow membrane pore diameter = 0.05 µm; outflow membrane pore diameter = 0.2 µm; inflow membrane diameter = 50 mm; outflow membrane diameter = 25 - 35 mm (X0.85).

Right hand side: System IIS (slow flow apparatus; flow rate 1.0 - 2.0 $cm^3 min^{-1}$); inflow membrane pore diameter = 0.03 µm; outflow membrane pore diameter = 0.2 µm; both membrane diameters = 25 mm; (X0.85).

PYREX
250ml

**PLATE : 2.03**

Forced ventilation apparatus (System IIF) for plant tissue culture with flow rate of 3.5 - 5.0 $cm^3min^{-1}$; inflow membrane pore diameter = 0.05 μm; outflow membrane pore diameter = 0.2 μm; inflow membrane diameter = 50 mm; outflow membrane diameter = 25 - 35 mm (X1.8).




❶ Inflow membrane
(pore diameter = 0.03-0.05 μm)

❷ Outflow membrane
(pore diameter = 0.2 μm)

❸ Inflow turret

❹ Outflow turret

❺ Water reservoir

❻ Inner hole

❼ Outer hole
(for injecting water)

❽ Silicone rubber bung

❾ Glass tube

mm; ID = 3.75 mm) opening into the humidified head-space of the inflow turret, just below the membrane directs the incoming gas flow into the culture vessel

Another glass turret, acts as an outflow turret, for the venting gases. The outflow turret is bounded by a larger-pored membrane (MPD = 0.2 μm). The pore size is large enough to allow reasonably free venting by both Poiseuille flow and diffusion, but sufficiently small to prevent contamination by bacteria and fungal spores. This turret is dry and provides the path of least resistance between the inflow turret and the atmosphere.

For throughflow ventilation, the culture vessel is fitted with a silicone rubber bung penetrated by two glass tubes (ID = 3.0 mm; OD = 6.0 mm). The longer tube (length = 90 mm) is connected to the inflow turret and the shorter one (length = 60 mm) to the outflow turret. Flexible PVC tubing (ID = 4.0 mm; OD = 5.5 mm) is used to connect the turrets to the tubes The evaporating water level of the inflow turret is always adjusted to within 2 mm of the membrane at the beginning of each day. The level tended to fall by 1 - 4 mm during a 24 h period, hence the necessity for re-adjustment.

The major disadvantage of System - I is the need to adjust daily the water level of the inflow turret to keep it as near to the membrane as possible. Another drawback of this design is that, as the turrets are connected to the culture vessel through flexible tubing, it is necessary to use two separate clamps to hold the inflow and the outflow turrets To overcome these drawbacks and to simplify the system, a new design was developed combining the inflow and outflow turrets into one unit.

### 2.2.2. System II :

The new model has been made in two sizes, one, System IIF, having a larger inflow membrane (D = 50 mm; MPD = 0.05 μm and producing a faster flow), and a smaller edition System IIS, having a smaller inflow membrane (D = 25 mm and MPD = 0.03 μm and producing a slower flow). The diameter of the outflow membranes for IIS is 25 mm, and for

IIF is 25-40 mm with similar MPD (0.2 μm) in each case. The dimensions of the larger version System IIF are described here.

This system is a single unit, consisting of an inflow and an outflow turret connected to each other with a silicone rubber bung (as shown in Fig 2.15). The inflow turret consists of a cylindrical double layered glass chamber (OD = 65 mm; ID = 45 mm; length = 82 mm). Inside the turret there is Oasis material which is highly porous (Plate 2.04), and able to absorb *ca.*98% of its own volume of water. The double walled chamber acts as a water reservoir and has 3 small holes (diameter = 5 mm). The outer hole, at the top of the outer wall, is used for injecting sterile water into the water reservoir by means of a hypodermic syringe. The water then passes through the inner holes at the bottom of the chamber and wets the Oasis, the water rising by capillary action and / or water pressure to the top of the Oasis. The polycarbonate Nuclepore membrane functioning as the inflow membrane (diameter = 50 mm; pore diameter = 0.05 μm; porosity 10%) is positioned centrally over a glass ring (thickness = 2 mm; diameter of the hole = 47 mm) and attached to the rim by silicone rubber compound. The ring supporting the membrane is fixed by silicone grease to the edge of the inflow turret. The gap, containing air, between the membrane and Oasis is called the head-space and is very narrow (<2 mm in depth). At the centre of the turrets there is a thin glass tube which opens to the head-space and directs the incoming gas flow to the culture vessel

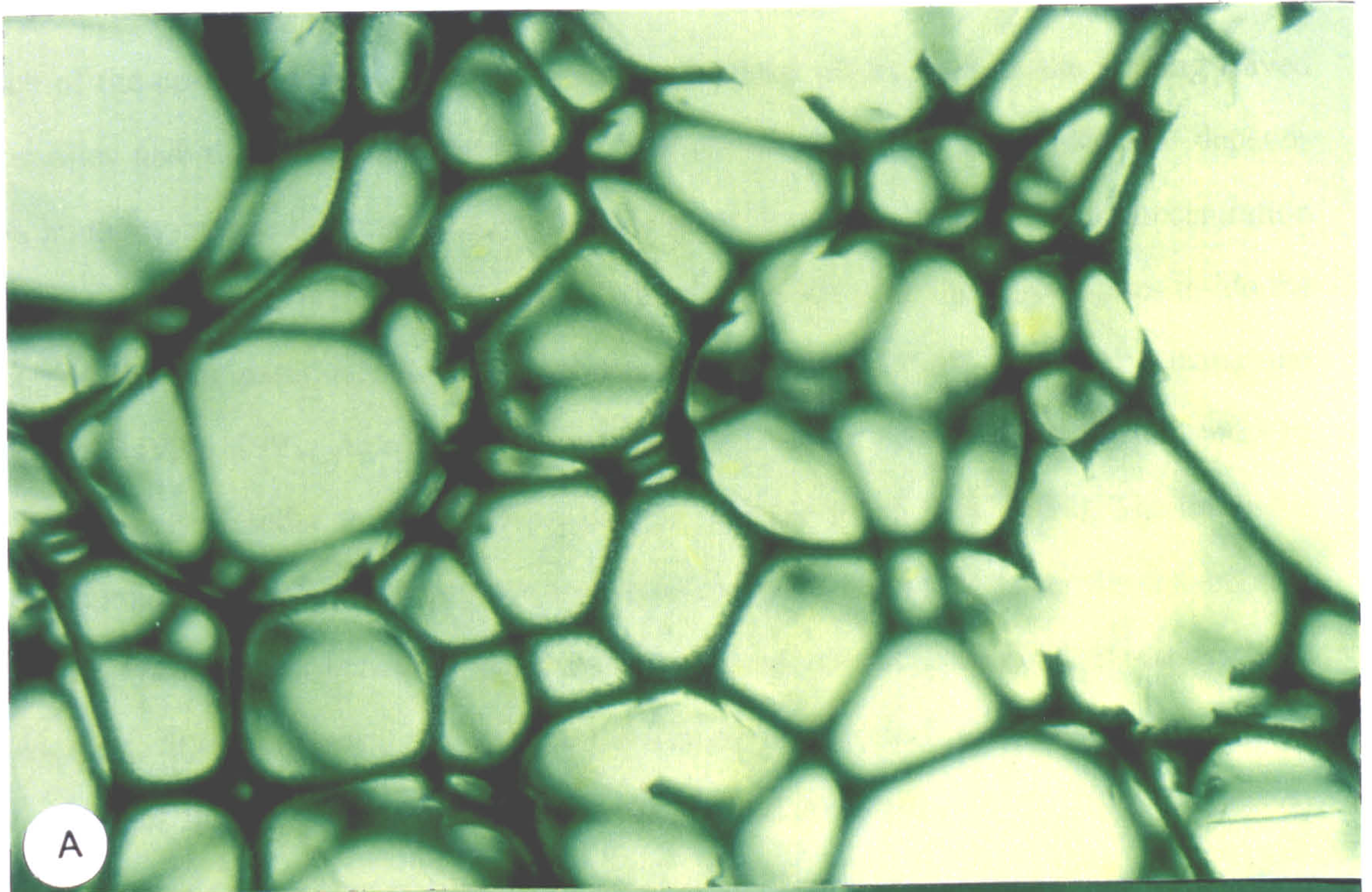
The outflow turret is also made of glass (OD = 48 mm; ID = 45 mm; length = 78 mm) and has a hole at one side (diameter = 21 mm). Another membrane, the outflow membrane (MPD = 0.2 μm; diameter = 25 - 40 mm; porosity = 70%) attached by silicone rubber compound across the 'mouth' of a specially made glass funnel (max. ID = 38 mm; length = 26 mm), is fixed to the hole of the turret through a silicone rubber bung. The elongated end (length = 35 mm; OD = 10 mm; ID = 7 mm) of the outflow turret enters the culture vessel through another silicone rubber bung. The gases flow from the culture vessel to the outflow turret through the narrow space between the glass tube and wall of the

**PLATE : 2.04**

Sections of oasis material photographed in green light :

above : low power to show highly porous material (X *ca.* 100).

below : high power with a porous membrane filling a small, only partially expanded gap on the structure (X*ca.* 1000).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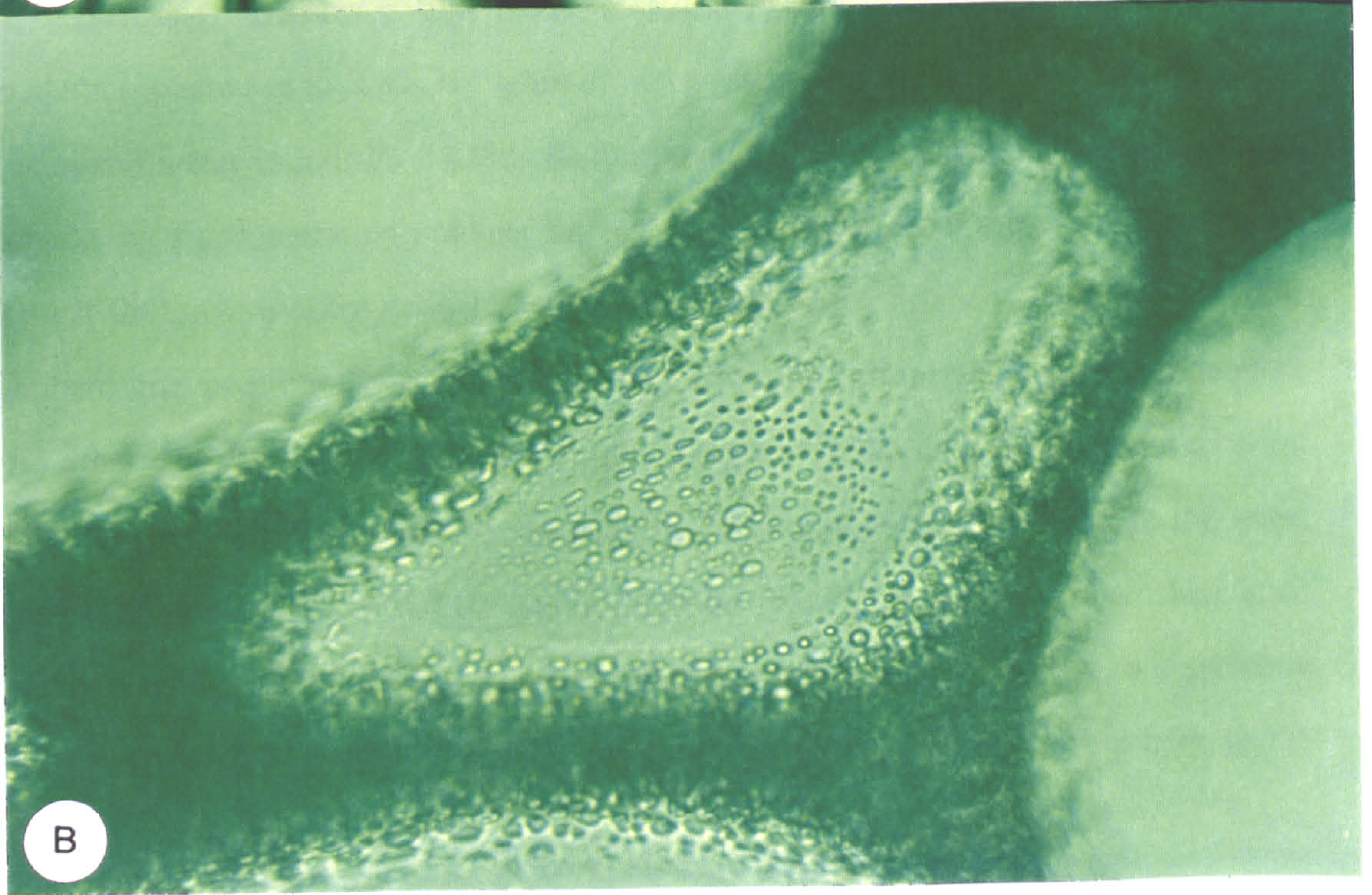

B

elongated end of outflow turret and are finally released to the atmosphere through the outflow membrane.

## 2.4. THE ORIGIN OF THE VENTILATION SYSTEM

Much of the convective gas-flow which aerates wetland plants such as the floating leaved water-lilies and the emergent macrophytes *Phragmites australis* and *Typha* sp. depends upon humidity-induced diffusion of air into the plant under the influence of a concentration gradient caused by the humidity differential which exists between the humid gases inside the plant and the comparatively dry atmosphere outside (Dacey 1980, 1981; Armstrong and Armstrong 1990, 1991; Armstrong, Armstrong & Beckett 1996a, Brix, Sorrell and Orr 1992; Tornbjerg, Bendix and Brix 1994; Bendix, Tornbjerg and Brix 1994). The lower the relative humidity in the atmosphere, the steeper is the gradient and the faster the convection.

In *Phragmites australis*, the humidity-induced convection (HIC) is driven by a diffusion of dry air across the stomatal surfaces of the leaf sheaths into the humid atmosphere of the sub-stomatal cavity (Armstrong and Armstrong 1990, 1991; Armstrong 1992; Armstrong, Armstrong and Beckett 1992). The constant humidification of the internal atmosphere (up to 2-3% by volume) within the gas spaces of the leaf sheath creates and maintains water vapour levels, thus diluting the atmospheric gases oxygen and nitrogen and producing a concentration gradient for their inward diffusion. If the stomatal resistance to inward diffusion is effectively less than any Poiseuille resistance to backflow to the atmosphere, the inwardly diffusing air will cause a pressurisation 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 like *Phragmites*, however, there is an alternative outflow path of low resistance, and the tendency to pressurise drives a convective flow (pressure flow) of gases into the underground parts from where they vent back to the

atmosphere through the broken ends of old dead flowering shoots - hence the term convective throughflow.

The ventilation system for plant tissue culture described here is based on the humidity-induced convection normally found in wetland species (e g. *Phragmites*) and provides a sterile flow of humid air to the culture vessel.

## 2.5. THE MECHANISM

The mechanism of the throughflow ventilation system is based on the principle of **"humidity-induced convection"**. For simplicity this process will be explained with reference to System I (Fig. 2.14), but it equally well applies to System II

The passage of atmospheric gases into the inflow turret takes place through a microporous partition, a Nuclepore membrane, whose pores (diameter = 0 03 - 0.05 μm) have a greater resistance to Poiseuille (pressurised) flow than to diffusion. The constant evaporation of water from the free-water surface (System I) or the saturated Oasis surface (System II) humidifies the head-space of the inflow turret and consequently dilutes the combined oxygen and nitrogen concentrations close to the water surface by up to 2-3%, and below the membrane by somewhat less than this. The result of this is the establishment of a concentration gradient for the inward diffusion of these gases across the membrane from the drier outer air. Since the membrane is very thin ($\leq$10 μm), the gradient can be very steep and the diffusion rate fast. The lower the RH of the ambient air and the closer the water surface is to the membrane, the steeper is the gradient for the inward diffusion of oxygen and nitrogen, and the faster is the flow. Although a more than reciprocal outward diffusion of water vapour takes place through the membrane, the water vapour is constantly replaced by evaporation Hence, since the membrane has a very significant resistance to any pressurised outflow, the inward diffusion coupled to the constant humidification creates a pressurisation of the gases occupying the head-space of the inflow turret. A glass tube opening into the humidified head-space of the inflow turret (Fig. 2.14) directs the pressurised incoming gases

to the head-space of the culture vessels and a flow will be maintained as long as (a) the RH of the outer air is <100%, and (b) internal evaporation continues to buffer the water vapour components of the head-space of the inflow turret. The latter depends upon the water availability in the Oasis material and availability of latent energy for evaporation. The venting of gases occurs via the larger pored membrane of the non-humidified outflow turret The pores (diameter - 0.2 μm) of the outflow membrane, are sufficiently large to allow reasonably free venting by both Poiseuille flow and diffusion, but small enough to prevent contamination of the system from bacteria and fungal spores.

It should be noted that if the escape of gases through the larger pored membrane could be prevented, the gases entering via the small pored membrane would create a substantial pressure, the static pressure $\Delta P_s$ ($\leq$ vapour pressure of water vapour beneath the membrane). Under flow conditions, i.e. with gases escaping via the larger pored membrane, a much lower pressure, the dynamic pressure $\Delta P_d$, is developed, just sufficient to drive the flows. Details are presented later (see Chapter III).

## 2.6. THE MATHEMATICS OF THE VENTILATION SYSTEM

As mentioned earlier the ventilation system is based on one of the pressurised gas-flow systems occurring naturally in plants, namely "humidity-induced convection", and the pressure and flow generation is driven by what has been termed humidity-induced diffusion (Armstrong *et al.* 1991). Since the flows can be generated by the use of physical models, the process is perhaps best explained in detail by reference to a simple model system (Fig 2.16). The figure shows sectional views through a cylindrical chamber having a micro-porous partition at one end, which at first (Figs 2.16a,b,c) is temporarily sealed from the atmosphere by a cover; passing through the base of the chamber is a venting pipe which may be closed by means of a tap. It is assumed that the pore diameters in the partition are very small and well within the Knudsen diffusion regime (Leuning 1983) e.g. << 0.1 μm, the mean free path length of the gas molecules. Such a partition, while allowing the diffusion of

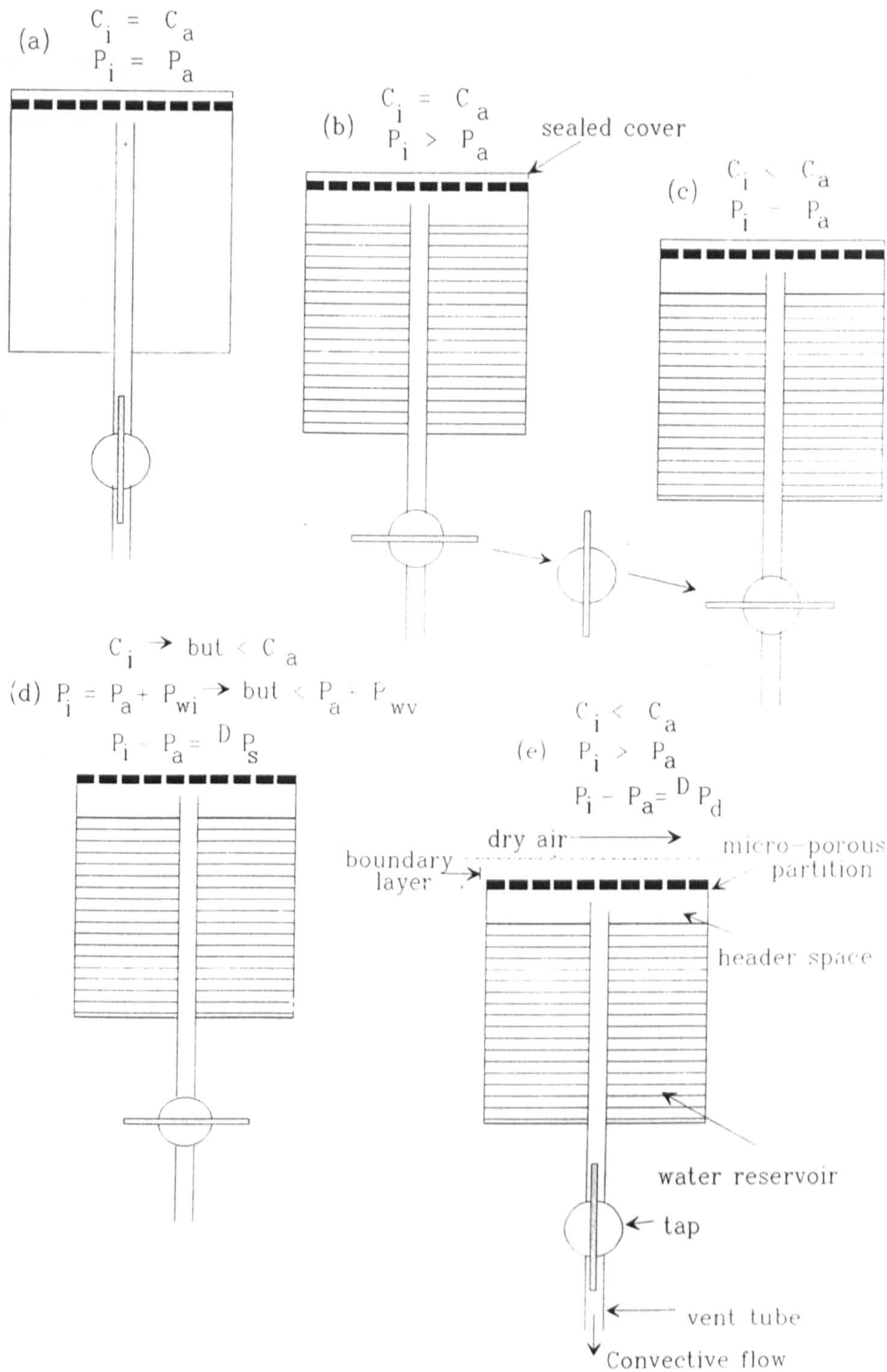



**Fig. 2.16.** Stages in the development of pressurisations and humidity-induced covective flows in a simple model by the process of humidity-induced diffusion across a microporous membrane. $C_i$ refers to the concentration of atmospheric gases (excluding water vapour) inside the chamber, and $C_a$ the concentration in the outside atmosphere. It is assumed that the atmosphere above the membrane is dry and the isothermal conditions prevail throughout. It should be noted, however, that convections would be produced even if the inside temperature ($T_i$) was lower than that outside ($T_a$), and if RH (relative humidity) was 100% inside and out, provided that $T_i > T_a$. $P_{wv}$ is the saturated water vapour pressure. For (a) - (e) see the text. (After Armstrong, Armstrong and Beckett 1996a).

gases through it, will effectively offer infinite resistance to pressurised flow (Armstrong, Armstrong and Beckett 1996a).

Details of the stages, which are outlined in Fig. 2.16, are as follows:

(a) If the chamber first contains dry air, is surrounded by an atmosphere of dry air at atmospheric pressure, is at the same temperature as its surroundings, and the tap open, it can be deduced that the concentrations of oxygen and nitrogen (plus the rare gases) within, $C_i$, and without, $C_a$, will be equal, i.e. $C_i = C_a$ and their percentage volumes inside and out will be 100%; similarly the total pressure inside and out will be the same, i.e. $P_i = P_a$.

(b) If water could now be introduced into the chamber without immediately entering the vapour phase, and the tap instantaneously closed, some water will subsequently enter the vapour phase. If the temperature control is such as to maintain $T_i = T_a$, then a situation will be created in which the concentrations of oxygen and nitrogen will be unchanged, i.e. $C_i = C_a$. However, due to the additional presence of the water vapour, the internal pressure will rise so that $P_i > P_a$ (Fig 2.16b). Since the system is presently gas-tight, the eventual value of $P_i$ will be $P_a + P_{wv}$, where $P_{wv}$ is the saturated water vapour pressure at that temperature, e.g. 2.337 kPa at 20°C.

(c) If the tap is now opened briefly to equalise the two pressures, and then closed once more, some gas will be vented and the new conditions will be $C_i < C_a$, and once more, $P_i = P_a$ (Fig. 2.16c). The amount of gas vented will approximate to the water vapour volume that had accumulated. At 20°C the combined percentage volume of the oxygen and nitrogen and rare gases ($C_i$) will now be 97.7% and the volume of water vapour 2.3%. In the dry air outside $C_a$ remains equivalent to 100%.

(d) If the seal is now removed from above the porous partition (Fig. 2.16d), then because - $C_a > C_i$, the external gases will diffuse into the chamber. Similarly water vapour will diffuse out, but, because of the water reservoir it will be instantly replaced. The diffusive entry of oxygen and nitrogen will thus once more cause an increase in $P_i$, and if partition pore size is such as to prevent a pressurised outflow, then as $C_i$ approaches $C_a$ so will $P_i$ approach (but

not reach) $P_a + P_{wv}$. At *equilibrium* $P_i - P_a$ will be what is termed the **static pressure differential**. If the atmosphere inside the chamber was totally saturated with water vapour, $P_i - P_a$ would be equal to $P_{wv}$. In practice, however, if water vapour can diffuse through the partition, then it could never reach complete saturation under the partition and, under isothermal conditions, water vapour this value of 2.337 kPa could only be approached and never fully realised.

(e) If the tap on the venting tube is now opened, there will be a rapid initial outflow of gas from the chamber and $P_i$ will fall. However, provided that the water vapour in the chamber is replaced by evaporation, the concentration of oxygen and nitrogen, $C_i$, will never reach $C_a$, and gases will continue to diffuse in across the partition to the extent that $P_i$ will remain greater than $P_a$, and the pressure differential $P_i - P_a$, termed the **dynamic pressure**, will continue indefinitely to drive gases through the venting pipe. The rate at which this will occur will be equal to the rate of inward diffusion across the porous partition, and will be a function of (i) the partition thickness, (ii) partition porosity, (iii) partition pore diameters, (iv) the concentration difference across the partition which is in turn a function of the water vapour concentration maintained beneath the partition, and (v) the venting path resistance. If the partition is very thin and highly porous, and provided that a high water vapour concentration can be maintained at the lower surface of the partition, very high rates of flow can be realised. It should be noted that a supply of heat from the surroundings is necessary to provide the latent heat of evaporation for the water. Without this source of energy the humidity gradient could not be maintained and no flow would occur.

Predictions of the static pressures and flows which can be generated in a system such as that shown in Fig. 2.16 can be made using a number of relatively simple equations (Armstrong, Armstrong & Beckett 1996a). For example it can be deduced that the static pressure differential generated (Fig 2.16d), although caused by the diffusive inflow of atmospheric gases as well as the replacement of any lost water vapour, will be numerically equal to that of the partial pressure of water vapour beneath the membrane. If the membrane

is 'non-leaky', i.e. the pores will not allow a pressurised backflow, this pressure differential can be determined by first estimating the water vapour flux, $J_{wv}$, through the membrane. If $R_h$ is the head-space diffusive resistance, $R_{md}$ the water vapour diffusive resistance of the membrane, and $R_b$, any boundary layer resistance, then:

$$J_{wv} = (P_{swv} / P_a) / (R_h + R_{md} + R_b), \tag{2.01}$$

where the saturated water-vapour pressure, $P_{swv}$, at 20°C at the water surface is 2.337 kPa, $P_a$ is 101.3 kPa, and 2.337/101.3 is therefore a concentration difference between the water surface and the dry air above the boundary layer (in fractional volume terms $m^3\ m^{-3}$), and $J_{wv}$ has units of $m^3\ s^{-1}$.

The partial pressure drop (in kPa) across the membrane itself ($\Delta P_{w(m)}$), numerically synonymous with the static pressure differential, will be:

$$\Delta P_{w(m)} = P_a\ (J_w \times R_{md}) \tag{2.02},$$

Similarly in Fig. 2.16e, the inward flow of atmospheric gases can be predicted. Since the presence of $H_2O$-vapour is at the expense of the other atmospheric gases, their partial pressure drop across the membrane will be equal in magnitude to that of the water vapour but in the opposite direction. This will generate an inward flow of atmospheric gases, $J_{o,n}$, into the chamber according to the equation

$$J_{o,n} = (\Delta P_{w(m)} / P_a) \times 1 / R'_{md} \tag{2.03},$$

where $R'_{md}$ is the diffusive resistance of the membrane to oxygen and nitrogen. If the venting tube is open and has no significant resistance, this flow will be sustained indefinitely and represents the maximum potential rate of convective gas flow. It should be noted that if pore sizes are $\leq$ 0.1 μm, the resistances $R_{md}$ & $R'_{md}$ will involve Knudsen diffusion coefficients ($D_K$). These will always be less than the normal mutual diffusion coefficient, $D_o$, and are determined from the following equation:

$$D_K = (d/3)\sqrt{[8RT/(\pi M_i)]} \qquad \text{(Leuning 1983) (2.04)},$$

where $M_i$ is the molecular mass of the diffusing species i. In Eq. 2 02 the Knudsen diffusion

coefficient for water vapour will be used, whereas in Eq. 2.03 it will be the average Knudsen diffusion coefficient for the gases oxygen and nitrogen appropriate to the pore diameters.

If pore diameters are $\geq 0.2$ µm and therefore outside the Knudsen regime, $R_{md}$ and $R'_{md}$, no longer depend upon pore diameter, only porosity, and they incorporate the use of the same mutual diffusion coefficient $D_o$, and are therefore equal. However, because the pores are outside the Knudsen regime any tendency to pressurisation in the chamber (e.g. Fig. 2.16d) will be counteracted by a pressurised backflow ('leakiness') through the membrane

The potential static pressure differential, $P_a(J_w \times R_{md})$ (equ. 2.02), will not now be realised; instead, there will be some lower value attained at which a diffusive inflow will become balanced by the Poiseuille backflow. This new pressure differential, the **effective** static pressure, $\Delta P_s$, can be determined using the following equation where the potential static pressure differential, $P_a(J_w \times R_{md})$ is represented as $\Delta P_{ps}$:

$$(\Delta P_{ps} - \Delta P_s)/ P_a \times 1/ R_{md} = \Delta P_s / R_{mp} \qquad (2.05)$$

where, for membranes with MPD $\geq 0.2$ µm, $R_{mp}$ is the Poiseuille Flow resistance.

The expressions used to determine Poiseuille Flow resistance, $R_{mP}$, of a porous partition (membrane) is:

$$R_{mP} = 8\eta L_m/(\varepsilon A r_1^2) \qquad \text{(Armstrong, Armstrong and Beckett 1988) (2.06)}$$

where, $\eta$ is the viscosity of air ($18.4 \times 10^{-6}$ kg s$^{-1}$ m$^{-1}$ = N s m$^{-2}$), $L_m$ is the thickness of the partition, $\varepsilon$ its fractional porosity, A its cross-sectional area, and $r_1$ is the radius of an individual pore. For the venting tube resistance, $R_{vP}$ the expression would be:

$$R_{vP} = 8\eta L/(\pi r^4) \qquad (2.07)$$

The expression $(\Delta P_{ps} - \Delta P_s)/ P_a \times 1/ R_{md}$, will be the diffusive inflow (m$^3$ s$^{-1}$) under the partial pressure gradient of atmospheric gases numerically equal to $\Delta P_{ps} - \Delta P_s$, while $\Delta P_s/R_{mp}$ will be the Poiseuille backflow (m$^3$ s$^{-1}$) at the resultant effective static pressure differential $\Delta P_s$.

To predict the convective flow rates in Fig. 2.16e, it is necessary to embrace the resistance, if any, to venting through the outlet tube and any attached flow-meter. If pores are within the Knudsen regime, an equation having a similar form to equation (8) may be used, but in which $R_{mp}$ is replaced by the resistance of the venting path, $R_{VP}$, and in which $\Delta P_s$ falls to become the dynamic pressure, $\Delta P_d$:

$$(\Delta P_{ps} - \Delta P_d)/ P_a \times 1/R'_{md} = \Delta P_d / R_{VP} \quad (2.08)$$

The convective flow, HIC, is then given by:

$$HIC = \Delta P_d / R_{VP} \quad (2.09)$$

If the inflow pore diameters are outside the Knudsen regime there will be two Poiseuille flow resistances acting in parallel, that of the inflow membrane - $R_{mP}$, and that of the venting path - $R_{VP}$. In Systems I & II - $R_{VP}$ incorporates the outflow membrane It is necessary, therefore, to determine first the resultant resistance to pressure flow, $\Sigma Rp$. This can be obtained from the relationship:

$$1/\Sigma Rp = 1/ R_{VP} + 1/ R_{mP} \quad (2\ 10),$$

and $\Sigma Rp$ is then used in place of $R_{VP}$ in equation 2.08, and $R_{md}$ (as used in equation 2.05) will replace $R'_{md}$. It should be noted that as $R_{VP}$ becomes very large, $\Delta P_d$ should approach $\Delta P_s$.

## 2.7. PUTATIVE FEATURES OF THE VENTILATING SYSTEM

The mathematical treatment outlined in the previous section makes it possible to predict the degree of ventilation possible with humidity-induced based pressure flow systems and explore the likely effects of altering specifications such as head-space depth in the inflow turret, partition thickness, partition porosity, partition pore diameters and the atmospheric conditions surrounding the apparatus. The few examples presented here illustrate (a) the relationship between head-space depth in the inflow turret and potential for pressurisation at a fixed pore diameter (b) the relationship between pore diameters and the flows and potential static pressures at a fixed turret head-space depth, (c) the effect of head-space depth on the

humidity of the convective gas-stream, and (d) effects of inflow membrane porosity on flows. In Chapter III the effects of some different specifications are explored experimentally.

### 2.7.1. The potential for pressurisation at a fixed pore diameter in relation to head-pace depth in the inflow turret

It is evident from Fig 2.17 that the distance between the evaporating water surface and the membrane, i.e. the head-space depth, can exert considerable influence on the degree to which the apparatus can pressurise as a result of humidity-induced diffusion. Since for any membrane, no matter what its pore size or porosity, the potential to pressurise is directly related to the flow which can be generated (Armstrong 1992), it follows that it will be most desirable to maintain as short a head-space depth as is physically possible. In practice it can be difficult to make this space narrower than 1 mm because of the dangers of wetting the membrane. This was particularly the case with System 1 because of its free water surface which could so easily be moved if the apparatus was disturbed. The use of the Oasis material in System II removed this problem making it safe to achieve a 1 mm head-space depth The slightly uneven surface of the Oasis material still means, however, that distances of less than 1 mm are not to be recommended.

### 2.7.2. Pore diameter, flow and static pressure at a fixed head-space depth

Using the guideline given section 2.6 and assuming (i) a head-space depth of 4 mm between the membrane and the evaporating water surface in the inflow turret, (ii) a boundary layer of still air of 700 μm above the membrane, (iii) a surrounding atmosphere of completely dry air, (iv) a membrane of diameter 20 mm, porosity 10% and thickness 10 μm, and (v) no venting resistance, the dependency of convective flows and static pressures (interrupted flow) on pore diameter are predicted to be as shown in Fig. 2.18a & b.

The results demonstrate some particularly interesting features concerning the processes of humidity-induced pressurisation and flow. Firstly, as pore size diminishes the potential to pressurise increases and the rise is particularly steep at pore diameters < 0.1 μm

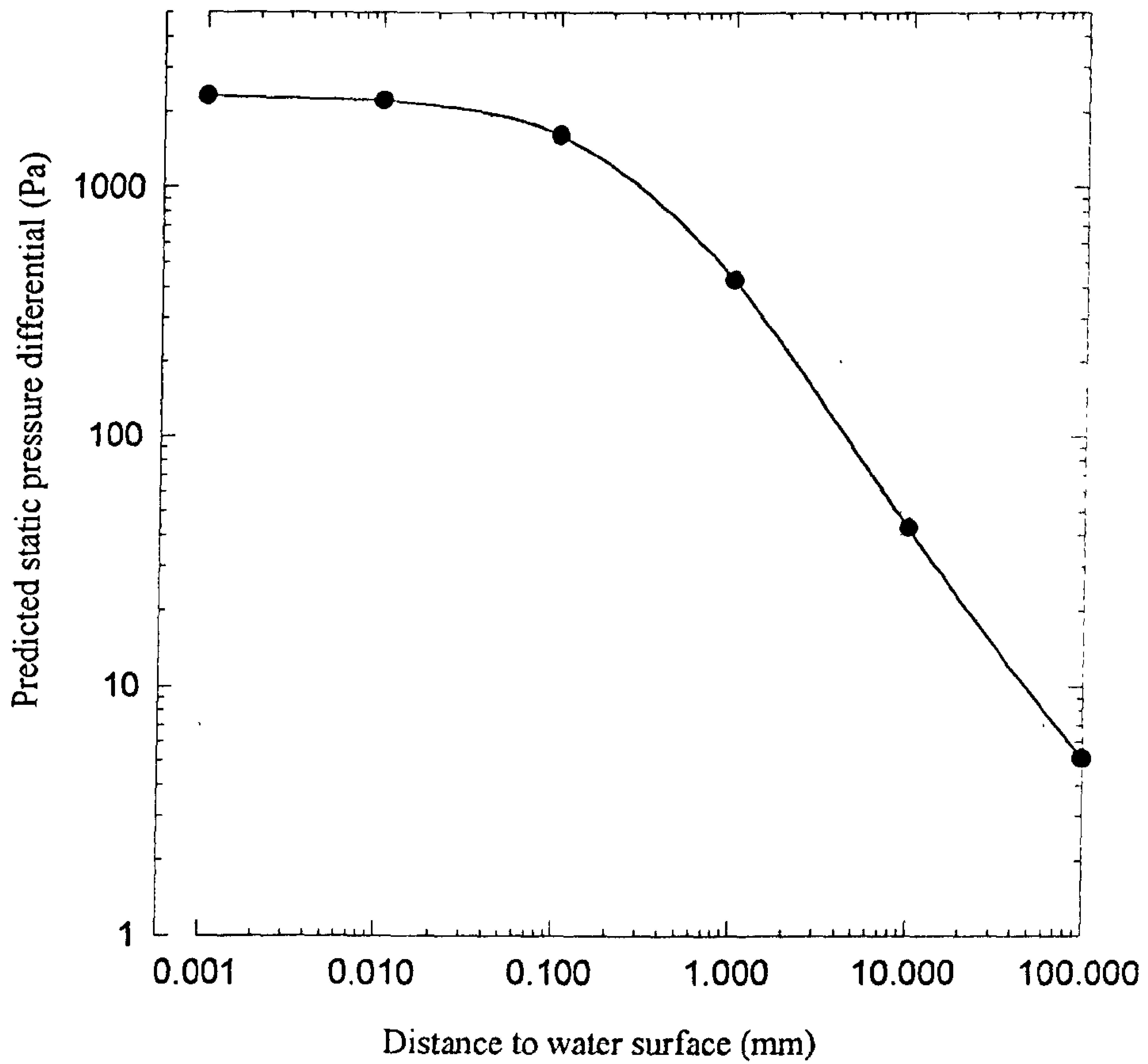



**Fig. 2.17.** Predicted relationship between static pressure differential and head-space depth for a membrane having pores of 0.046 μm diameter. Assumptions were: T = 20°C; membrane porosity = 10%; boundary layer thickness = zero. (Modified from Armstrong 1992).

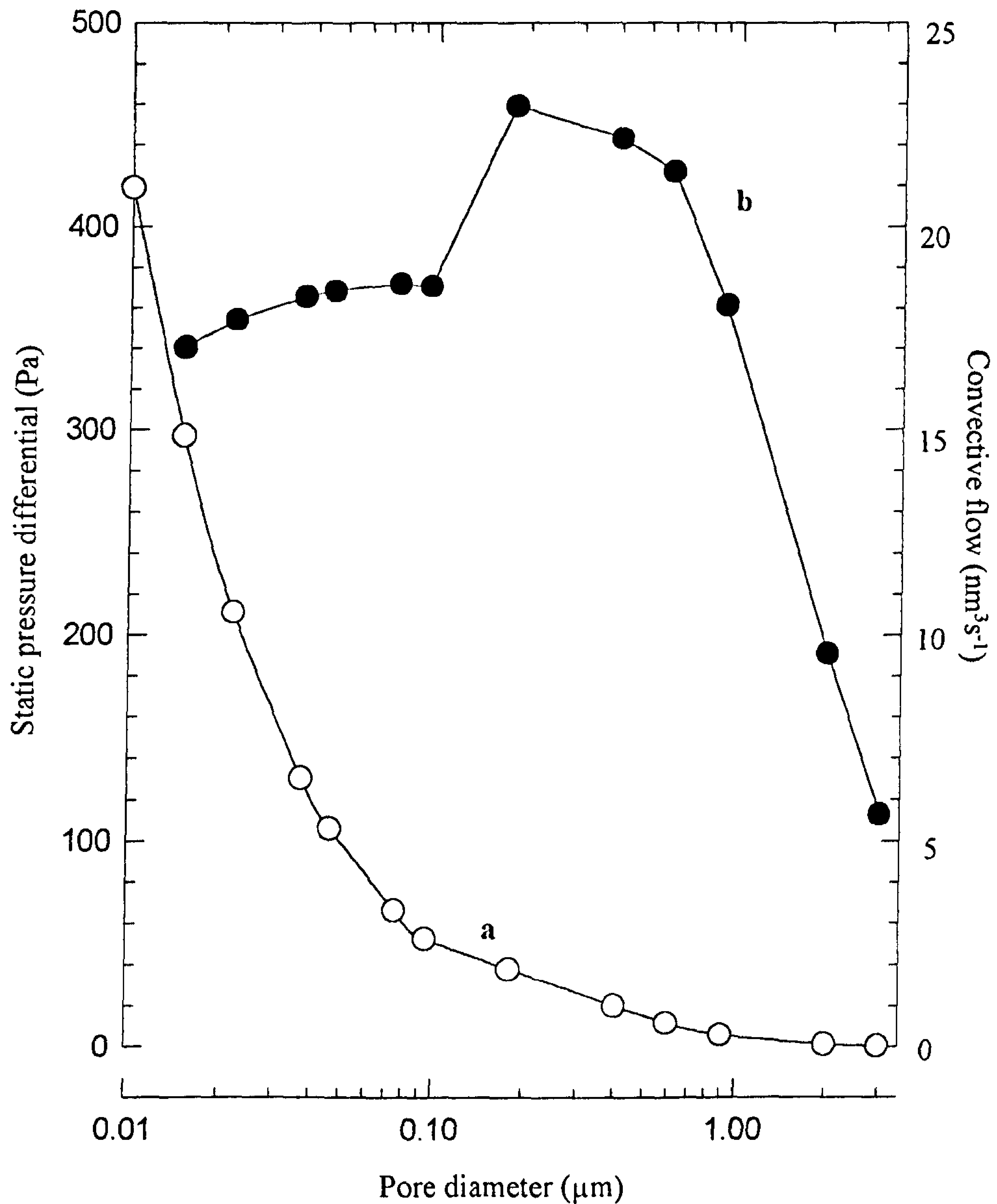



**Fig. 2.18.** Predicted relationships between (a) static pressures and pore diameter, and (b) convective flow generation and pore diameter. The data assume a head-space depth of 4 mm, a boundary layer thickness of 700 μm, a membrane diameter of 20 mm; porosity of 10% and thickness of 10 μm, and temperature of 20°C. (Modified from Armstrong 1992).

The value is approximately that of the mean free path length of diffusing gas molecules at normal pressures; below this is the Knudsen regime where the molecules will hit the pore walls more frequently than another gas molecule. Here diffusion rates are slowed and pressure flow is drastically reduced or prevented. Secondly, as a consequence of the lower diffusivities in the Knudsen regime it can be seen that the potential for convective flow diminishes at pore diameters below 0.1 μm. The third notable feature is that as pore size increases beyond 0.2 μm, flows diminish again. This is a function of membrane leakiness, i.e. the membrane allows more pressurised backflow because the holes are now big enough for more molecules to be involved in inter-molecular collisions.

For the construction of flow systems such as System I and II it is desirable that the inflow turret should have an inflow membrane capable of inducing as fast a flow as possible while at the same time developing a sufficient pressure to drive gas through the outflow membrane. On the other hand the outflow membrane should have as little resistance to pressure flow and diffusive flow as possible. At the same time both membranes should help maintain sterile conditions in the culture vessels. The results in Fig. 2.18 show that for a membrane with a porosity of 10%, (the standard adopted by Nuclepore, Millipore etc.), the flow and sterility conditions will be optimal with inflow membrane pore diameters of *ca.* 0.05 μm, and outflow diameters of 0.2 μm.

### 2.7.3. Membrane porosities

Pressurisation and flow are both influenced by membrane porosities. Clearly flows will be optimised if the outflow membrane offers as little resistance to diffusive- and pressure-flows as possible, and thus the higher the porosity the better. As regards the inflow membrane there is also a need to induce sufficient pressurisation to drive the flows, although it must be noted that provided the venting path has a very low resistance, not much pressure would be required to drive the flows. The results shown in Fig. 2.19 show that at an inflow membrane porosity of 10 % the convective flow is already reaching an asymptote and that potential pressures are more than adequate to drive appreciable flows in a low venting resistance

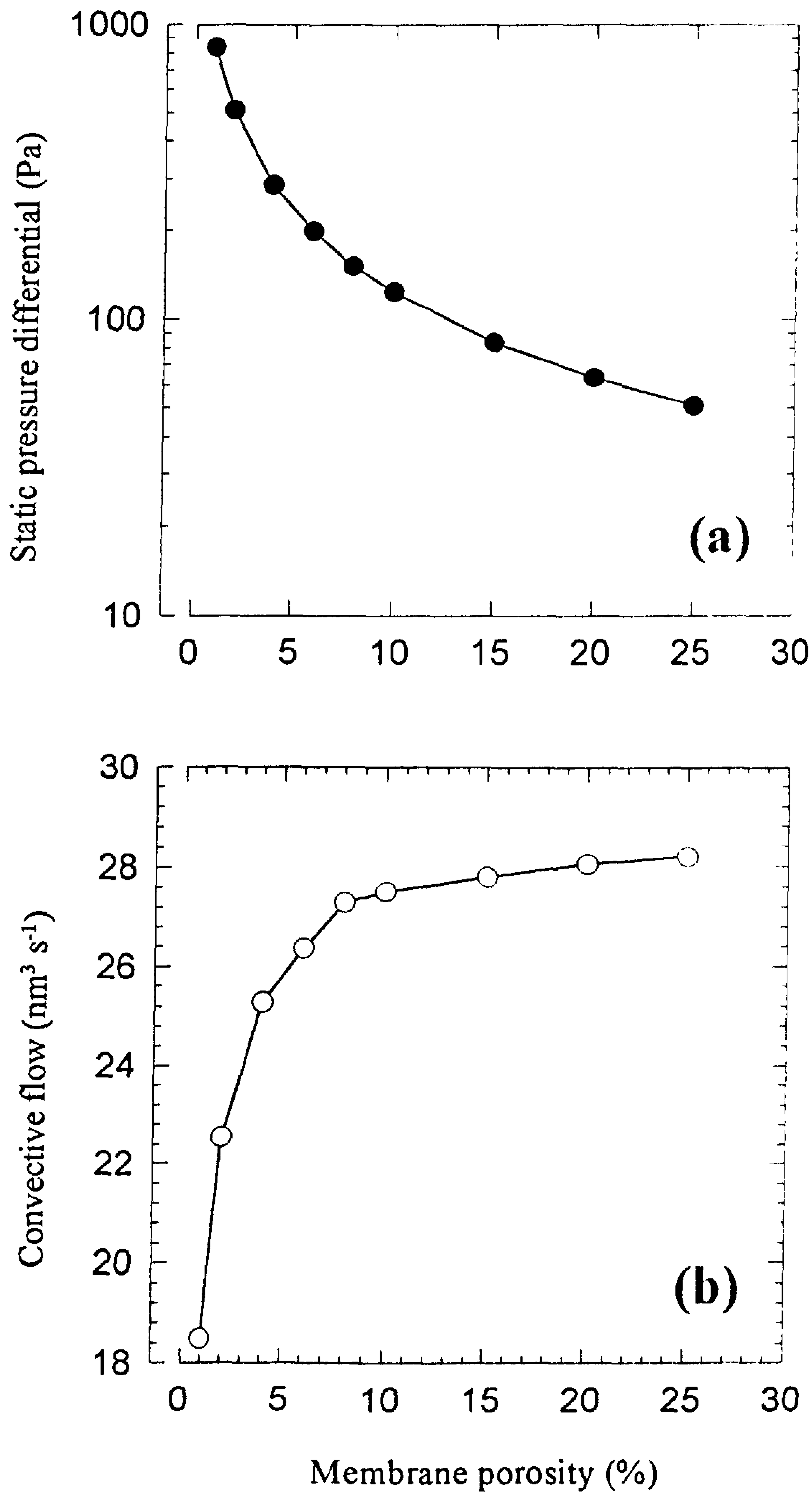



**Fig. 2.19.** Predicted effects of membrane porosity on (a) static pressures, and (b) convective gas-flows. Assumptions were: membrane diameter 20 mm; pore diameters = 0.046 μm; head-space depth = 4 mm and T = 20°C. No boundary layer was programmed. (Modified from Armstrong 1992).

### 2.7.4. Humidity of the convective gas stream

Estimates of the relative humidity of the convective gas-flows coming from inflow turrets are possible using the equations in Section 2.6, and some examples are shown in Fig 2.20 Both plots show that the humidity in the gas-stream should diminish as the head-space depth between the membrane and the evaporating water surface increases; further extrapolation would show, however, that the humidity could never fall below 50% RH. By reducing the distance between the membrane and the evaporating water surface it can be seen that the relative humidities could in theory be raised to approach 100%. The depth to the Oasis material in the turrets of Systems IIS & F was usually set at approx. 1-2 mm and hence, even with an external RH of 0%, the relative humidities in the convective gas streams should not fall lower than 55% By comparing plots (i) and (ii) in Fig. 2.20, it can be seen that higher external humidities will lead to higher humidities in the flows delivered by the inflow turrets. Thus if the relative humidity immediately above the inflow membrane were to be raised from zero to 35%, convective flow humidity should rise from 55 to 72%. Any boundary layer above the membrane would raise the humidity still further and so it can be expected that in practice under forced ventilation, vessel humidities should usually exceed 72%.

### 2.7.5. Membrane areas

Provided that the head-space diameter matches the diameter of the inflow membrane, it follows from the mathematical treatment in Section 2.6 that flow will be directly proportional to membrane area. Flows can thus be increased substantially by increasing inflow turret diameters; however, for ease of handling a limit must eventually be reached and it can be simpler to connect inflow turrets in parallel to achieve faster flows.

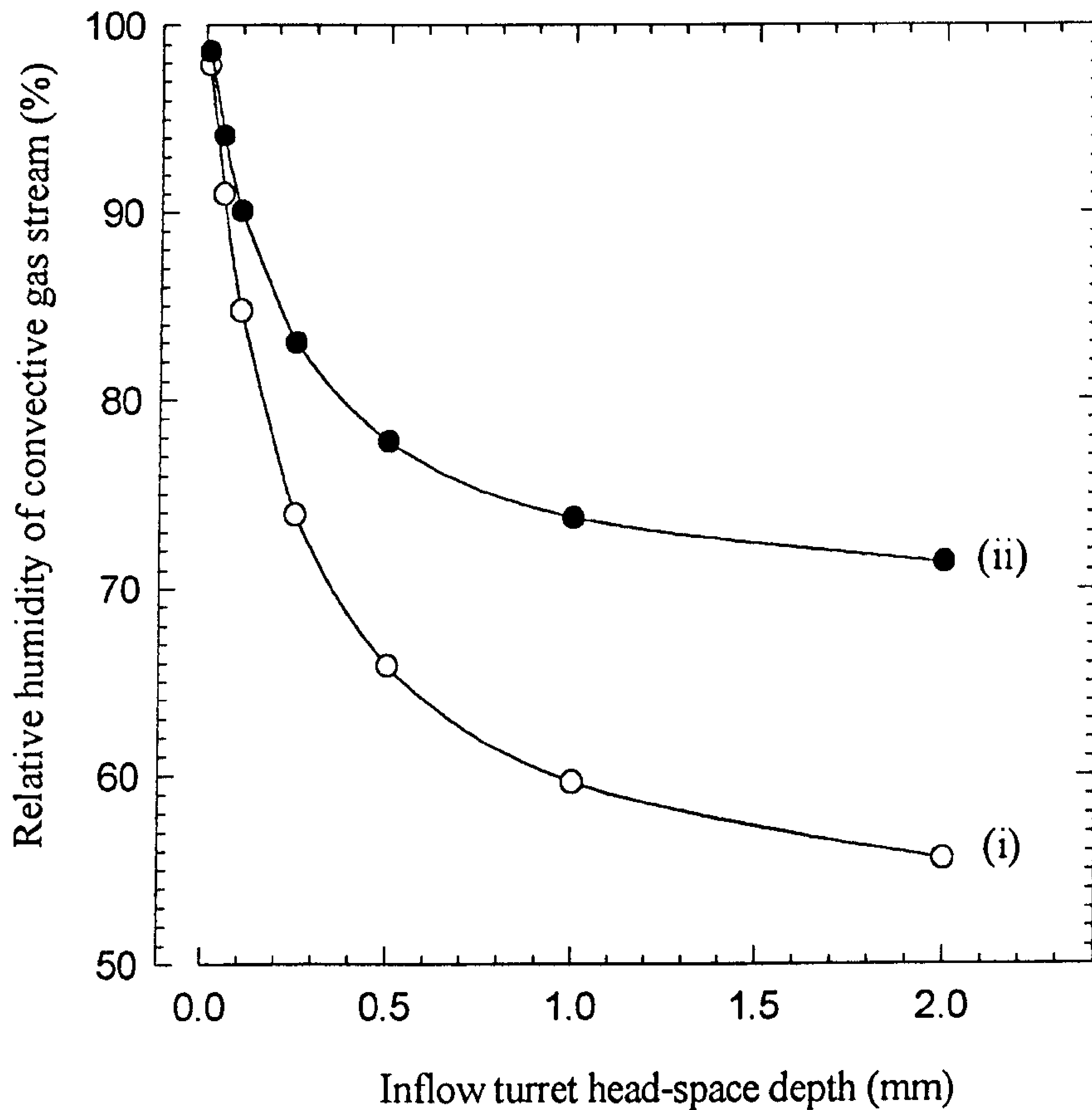



**Fig. 2.20.** Predicted humidity of the gas stream delivered from the inflow turret as a function of head-space depth to water surface, assuming that (i) dry air impinges on the upper surface of the inflow membrane, (ii) humidity of air on the upper surface of inflow membrane is 35% (RH).
The thickness of the inflow membrane has been taken as 10 μm, the pore diameters, 0.046 μm, and porosity, 10%; the ambient temperature has been assumed to be 20°C; no account has been taken of boundary layer thickness.

## 2.8. FINAL COMMENTS

The results presented above have briefly outlined some of the properties of the new apparatus on which this thesis is based. It is not within the scope of this thesis to explore fully the limits of the system, but in Chapter III experimental examples are presented; it will be seen that some of these compare closely with the predictions made above. It is clear, therefore, that further exploration of the systems' potential could readily be achieved by mathematical modelling based on the equations presented in Section 2.6.

# CHAPTER - III

## THE PERFORMANCE OF THE VENTILATION SYSTEMS

### 3.1. INTRODUCTION

Convective pressurised flows of gases, static and dynamic pressure differentials ($\Delta P_s$ and $\Delta P_d$), and other functional characteristics of the ventilation systems I and II are studied in this chapter. The static pressure differential ($\Delta P_s$), equivalent to the tendency for gases to enter the system, is the maximum pressure developed when the inflow chamber (turret) is connected only to the pressure transducer (non-throughflow condition). On the other hand, dynamic pressure, $\Delta P_d$, the pressure driving the convected gases through the system during ventilation, is measured when convective flow is taking place by having the pressure transducer connected laterally to the flow path (i.e. in parallel).

Some experiments were performed to estimate the effects of the following on the ventilation rates and in some cases on $\Delta P_s$ and $\Delta P_d$ of one or both of Systems I and II (Chapter II, Section 2.3): distance between the inflow membrane and evaporating surface (inflow head-space depth), interbatch variations of membranes, the presence and absence of agar and plantlets in the culture vessels, membrane pore diameters, exposed membrane area, wind speed and RH of the wind above the inflow membrane and resistance within the venting pathway. For both systems, the sustainability of flow rates without replenishment of water was also investigated.

Additionally the effects of sealed, diffusive and forced-ventilation systems were investigated in relation to the humidity within the vessel, and the $t_{50}$ values for the retention of injected ethylene examined in relation to these different ventilation systems.

## 3.2. MATERIALS AND METHODS

The convective flows (HIC), $\Delta P_s$ and $\Delta P_d$ differentials were studied by using the throughflow ventilation systems (Systems I and II) in a variety of ways i.e. varying the distance between the underlying water surface and membrane, increasing the resistance to venting, using different membrane areas, varying dry-air wind speed and the humidity of the wind across the inflow membrane.

A soap film flow-meter (length 400 mm; bore 5 mm) placed in series in between the inflow turret and the culture vessel (Fig. 3.01), was used to measure the humidity induced convective flow (HIC) of System I. A pressure transducer (Furness Controls Limited) was connected to the inflow turret by means of a three-way tap to measure the static pressure and dynamic pressures. Flexible PVC tubing (OD = 5.5 mm; ID = 4.0 mm) was used to connect the turrets to the flow-meter and the pressure transducer.

Static pressures and HIC flow rates of the two variants of System II were also measured. Again the flow meter (length 400 mm; bore 5 mm) was placed in series between the inflow turret and the culture vessel, and the central tube of the system which is normally used to direct the incoming gas, was connected to the flow meter and also to the pressure transducer through a three way tap (Fig. 3.02). An extra glass tube (length = 30 mm; OD = 10 mm) with a side arm (as shown in Fig. 3.02) was connected at the elongated end of the outflow turret. The side arm could then connected to the other end of the flow-meter with a flexible tubing for measuring flows in the completely assembled system.

## 3.3. EXPERIMENTS

Experiments 3.3.1 to 3.3.4 and 3.3.8 to 3.3.10 were performed with the turrets exposed to ambient conditions of constant RH and temperature (measured using a humidity/temperature probe - Vaisala, Sweden). Experiments (3.3.5) - (3.3.7) were performed with the inflow membrane exposed within a wind tunnel (Fig. 3.03) into which

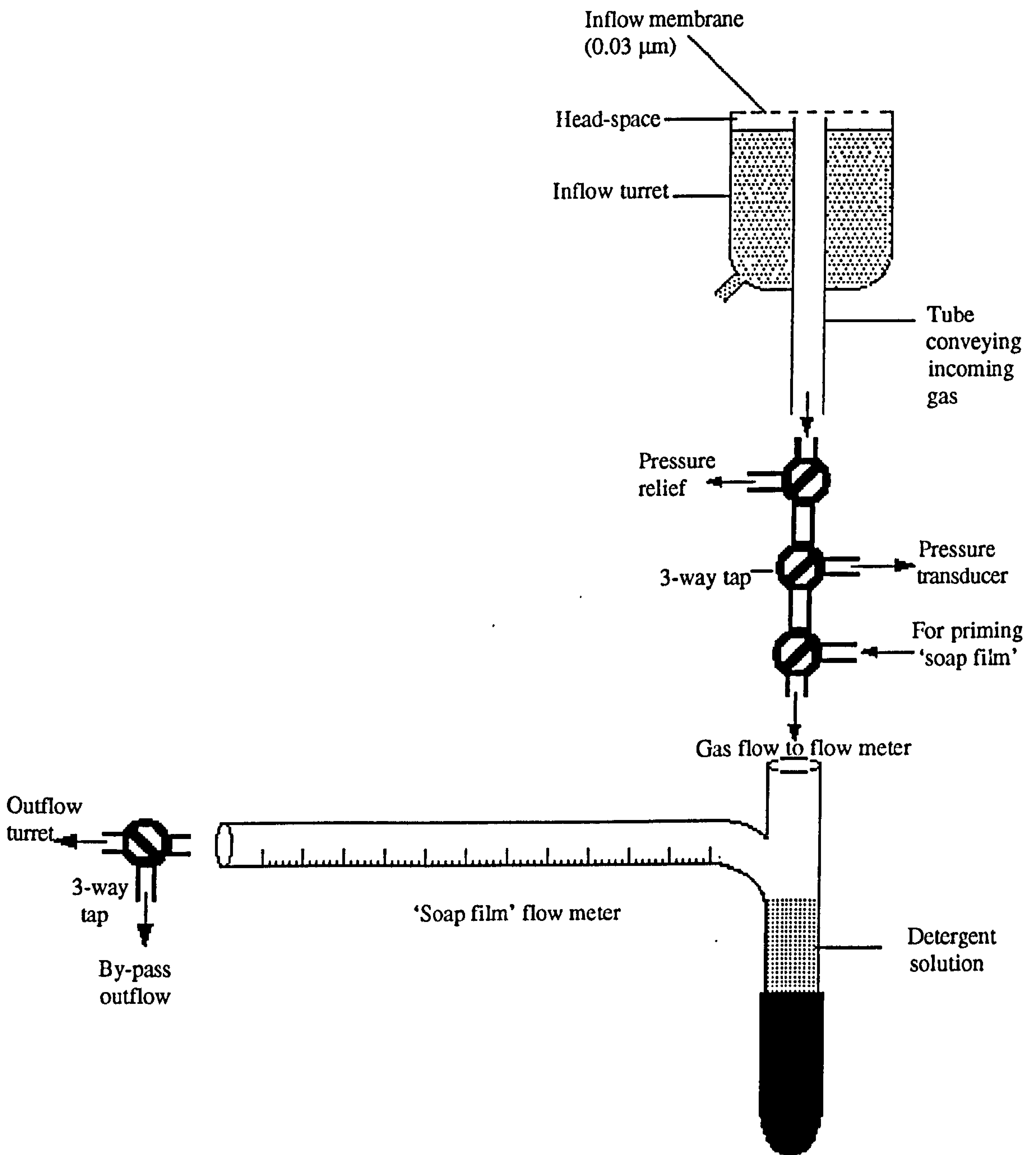



**Fig. 3.01. System I: a plan of apparatus for measuring flow rates, static pressure ($\Delta P_s$) and dynamic pressure ($\Delta P_d$) of the ventilation apparatus.**

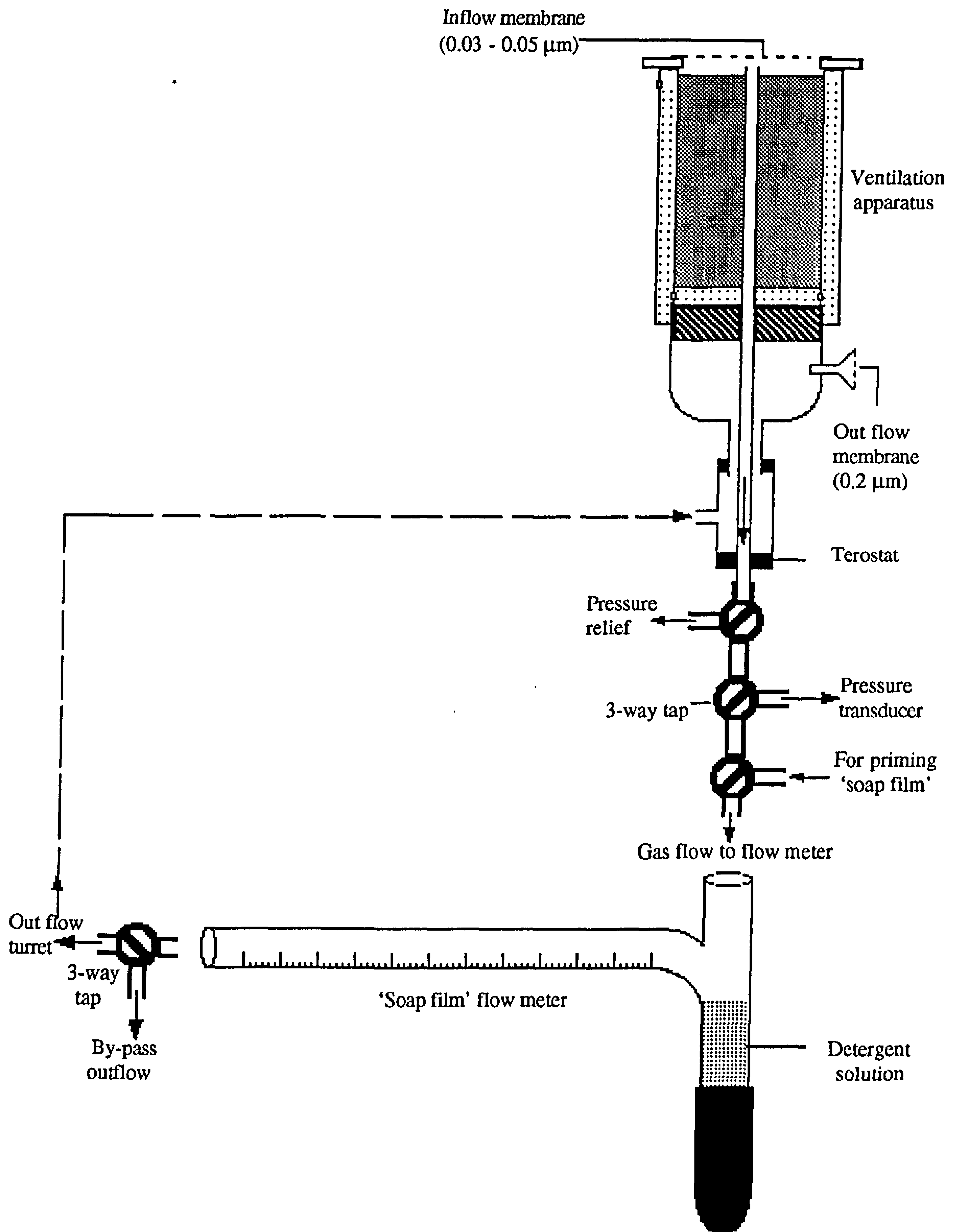



**Fig. 3.02. System II: a plan of apparatus for measuring flow rates, static pressure ($\Delta P_s$) and dynamic pressure ($\Delta P_d$) of the ventilation apparatus.**

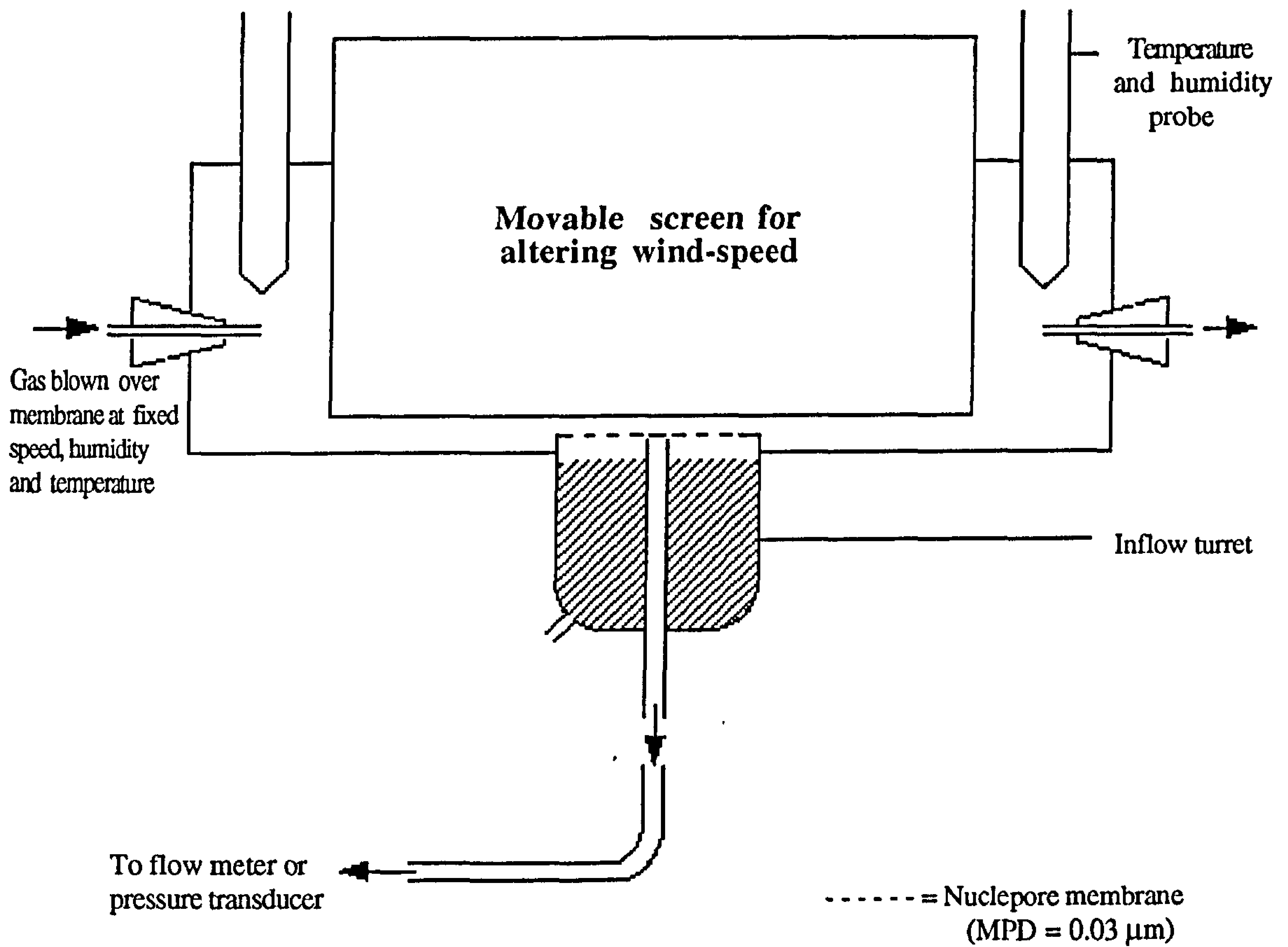



**Fig. 3.03. Apparatus for investigating humidity-induced convection (HIC), static pressure ($\Delta P_s$) and dynamic pressure ($\Delta P_d$) of the ventilation apparatus (System I) using the wind-tunnel for altering the wind speed over the inflow membrane.**

air at various humidities and speed ranges was delivered from an ADC humidity generator and compressed air cylinder.

### 3.3.1. Effects of varying distance between membrane and evaporating water surface.

Humidity-induced convection (HIC) and static pressure ($\Delta P_S$) values were measured with both Systems I and II for different distances (W) between the inflow-membrane and the evaporative water surface (head-space depth). The head-space depth of System I was varied over the range of 1.5 - 19.0 mm for a membrane with MPD of 0.03 μm. HIC and $\Delta P_S$ were recorded each time. The RH and ambient temperature were also recorded during the experiment.

Flow rates and static pressures ($\Delta P_S$) values of Systems IIF (Fast Flow) and IIS (Slow Flow) were also measured (i) for different head-space depths (varied from 1.5 - 31 mm) both with and without any Oasis material inside the inflow turret and (ii) for different distances between the inflow membrane and the Oasis.

In each of these experiments no outflow membrane was fitted.

### 3.3.2. Within batch variations of inflow and outflow membrane

The advertised percentage porosity for Nuclepore membranes used in System I was 10%. However, previously, different batches of membranes had been found to vary in this respect (Armstrong 1992).

This experiment was performed to examine any variation in the ventilation rates produced by different membranes of the same pore size, and the $\Delta P_d$ values. HIC flow rates and $\Delta P_d$ values were measured for different combinations of inflow and outflow turrets. Each of four different inflow turrets (MPD = 0.03 μm) was combined with each of four different outflow turrets (MPD = 0.2 μm; W = 2 mm; RH = 38%; T = 20 °C).

### 3.3.3. Effects of presence or absence of agar or plantlets

Culture vessels were capped with either the large (System IIF) or small (System IIS) ventilation apparatus, and flow rates were measured by connecting the flow meter in series between the culture vessel and the outflow turret (Fig. 3.02). The culture vessels were (a) empty, or (b) contained 10 $cm^3$ agar (depth = 8 mm), or (c) 10 $cm^3$ agar with an *in vitro*-grown tobacco plantlet (FW= *ca.* 300 mg; leaf area *ca.* 9.0 $cm^2$).

### 3.3.4. Effects of exposed membrane area

The standard inflow membrane areas of the ventilation apparatus were made progressively smaller by covering with Sellotape. Flow rates and static pressure values were measured for different inflow membrane areas of Systems I, IIS (slow flow) and IIF (fast flow). In each case a standard 25 mm diameter Nuclepore membrane (MPD = 0.2 μm) was used on the outflow turret. In the case of Systems I and IIS, in order to start with relatively high membrane areas, two inflow turrets were connected in parallel on the delivery side.

Humidity induced flow rates were also measured for different outflow membrane areas using System IIF capped with a Duropore membrane (thickness = 100 μm) having a porosity of 70% and an MPD of 0.2 μm.

### 3.3.5. Effects of pore diameter

Here the membrane end of the inflow turret (System I) projected through a neatly fitting hole into a Perspex "wind" tunnel (Fig 3.03), so that a stream of dry air could be passed at a fixed speed over the membranes of various pore diameters. Flow rates and ΔPs were measured for the inflow turret, System I: MPD = 0.015 μm - 3.0 μm; W = 4 mm. The wind speed of dry air across the membrane was 0.024 m $s^{-1}$; T = 19.4°C; RH = *ca.* 0% in wind tunnel.

### 3.3.6. Effects of varying wind speed of dry air across the membrane

The inflow turret of System I projected into a wind tunnel as in section 3.3.5. Through this, air at different wind speeds was blown horizontally over the membrane to develop a range of humidity gradients vertically across the membrane by varying the boundary layer thickness. Flow rates and static pressure values were measured for each value of wind speed (0 - 67 mm $s^{-1}$) across the inflow membrane of the ventilation apparatus. W = 4 mm; T = 19.4°C; RH = 0% in wind tunnel.

### 3.3.7. Effect of varying the humidity of wind across membrane

The inflow turret of System I was placed in the wind tunnel (as in the previous experiment Fig. 3.03) in a constant velocity air stream at constant temperature and at a range of humidities from 0% to 54% (by using an ADC humidity generator). Flow rates and static pressure values were measured for different humidities (W = 4 mm; MPD = 0.03 μm). Wind velocity was 0.056 m $s^{-1}$.

### 3.3.8. Effects of increasing the resistance to venting

Using both System I and System IIF and S, HIC flow rates and ΔPd values were measured for different resistances to outflow, by using different Nuclepore membranes (MPD = 0.03, 0.1, 0.2, 0.4 μm) as outflow membranes and also by introducing soap bubbles in series within the flow-meter.

HIC flow rates and ΔPd values were also measured for different resistances to venting by placing microcaps (each of 1 μl volume) in series between the exit tube and the flow meter.

### 3.3.9. Sustainability of flow rates

Both System I and System IIF (5 replicates of each) were set up under growth room conditions (ambient temperature 23°C; RH = 30$\pm$5 %) each being initially 'full' of water. Flow rates were measured every 24 h. The drops in water level of the water reservoirs

were also measured each day. This experiment was done in order to compare the duration of flow in the two systems without topping them up with water.

### 3.3.10. Estimation of humidity in the culture vessel with the sealed, diffusive and forced ventilation systems

Culture vessels (60 $cm^3$) were 'capped' in various ways: (a) with the forced ventilation apparatus (System IIF; flow rate 5 $cm^3$ $min^{-1}$), (b) with a conventional polypropylene membrane (thickness = 25 μm) and (c) with a silicone rubber bung.

The RH (%) of the culture vessels in each case was determined by inserting a humidity probe (Vaisala) into the culture vessel through the side arm.

### 3.3.11. Effects of different types of 'capping' of culture vessels on retention times, $t_{50}$, for injected ethylene

Glass culture vessels (60 $cm^3$) were 'capped' in various ways :

(a) by System IIS; dry turret - producing only diffusive ventilation; (b) by System IIS - slow flow (flow rate 1.0 $cm^3$ $min^{-1}$); (c) by System IIF; dry turret - producing only diffusive ventilation; (d) by System IIF - fast flow (flow rate 5.0 $cm^3$ $min^{-1}$); (e) by a conventional polypropylene membrane (thickness = 25 μm); (f) by a silicone rubber bung.

The side arm was sealed with a silicone rubber 'Suba-seal' to allow ethylene samples to be withdrawn by means of a hypodermic syringe. Silicone rubber was used since it does not itself produce ethylene although it will, to a certain extent be leaky to ethylene. Four of each type of assembly were used as replicates, and a 23 μl $l^{-1}$ ethylene/air mixture were injected through the 'Suba-seals' of the side arms of the assembly. Ethylene concentrations were then determined at regular intervals by removing 500 μl samples of gas from the assemblies and analysing by means of gas chromatography (PYE Unicam). A Poropack Q column (60/80 mesh) was used in a glass column (2438 mm X 0.64 mm) and column, injector and flame ionisation detector temperatures were 100, 150, and 150°C

respectively. The peak was identified by a retention time of about 1.4 min. Nitrogen was used as the carrier gas at a rate of 60 $cm^3min^{-1}$. The time taken for an assembled container to lose 50% of its content of ethylene gas ($t_{50}$) was determined from plots of ethylene concentration against time.

## 3.4. RESULTS AND DISCUSSION

### 3.4.1. Effects of varying distances between the inflow membrane and water surface

**System I**

The distance between the membrane and underlying water surface (W) in the inflow assembly has a very great influence on the magnitude of the static pressures and convective flows. It should be noted that the membrane could be easily wetted accidentally if the water level in the chamber is raised too high ($W \leq 1.0$ mm); therefore it was not possible to move the water level closer than 2 mm from the membrane. Static pressures and HIC flow rates increased as the distance between membrane and water level decreased. Maximum static pressures and HIC flow rate were recorded when W = 2.0 mm (static pressure 140 Pa; HIC flow rate = 18.4 $nm^3\ s^{-1}$) (Fig 3.04b). The average ambient temperature and RH during the experiment were 26°C and 19.6 % respectively.

**System II**

In the absence of any Oasis material in the inflow assembly the distance between the membrane and the free water surface (W) has a very great influence on the magnitude of static pressure and convective flows. Static pressure and HIC flow rates increased as the distance between membrane and water level decreased. Maximum static pressure and HIC flow rates were recorded when W = 1.75 mm (static pressure 62 Pa; HIC flow rate 150 $nm^3\ s^{-1}$ *ca.* 9 $cm^3\ min^{-1}$) (Fig 3.04a). This is a much higher rate than is normally obtained with System IIF but in this experiment external humidity was particularly low (18.5%), the ambient temperature relatively high (27°C), and the water surface domed so that the head space depth may have been effectively even less than 1.75 mm. A high temperature increases the atmospheric water vapour content of the inflow turret atmosphere; both this and a low external humidity increase the humidity differential across the membrane and hence the concentration gradient for the inward diffusion of oxygen and nitrogen to drive the convective flow.

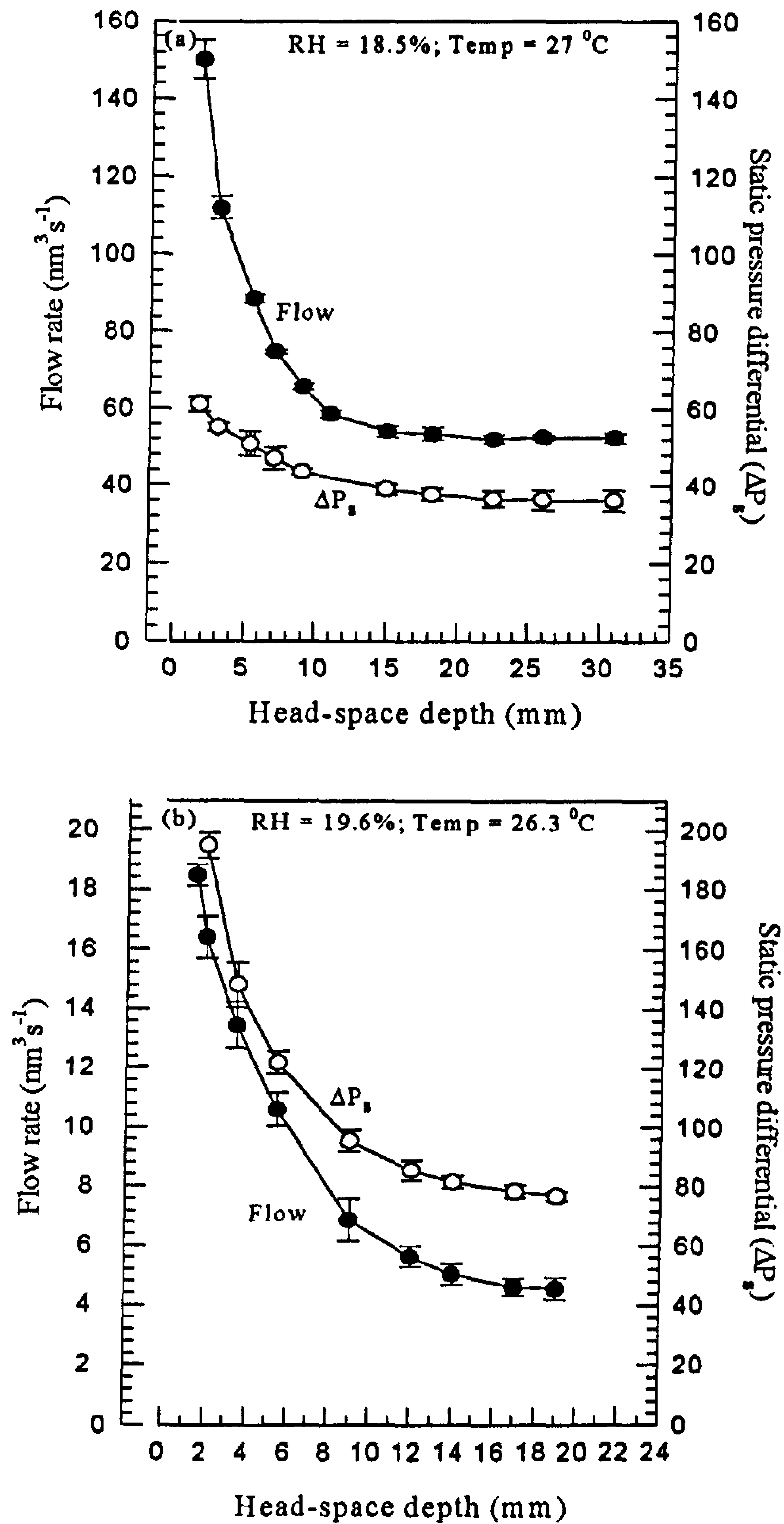
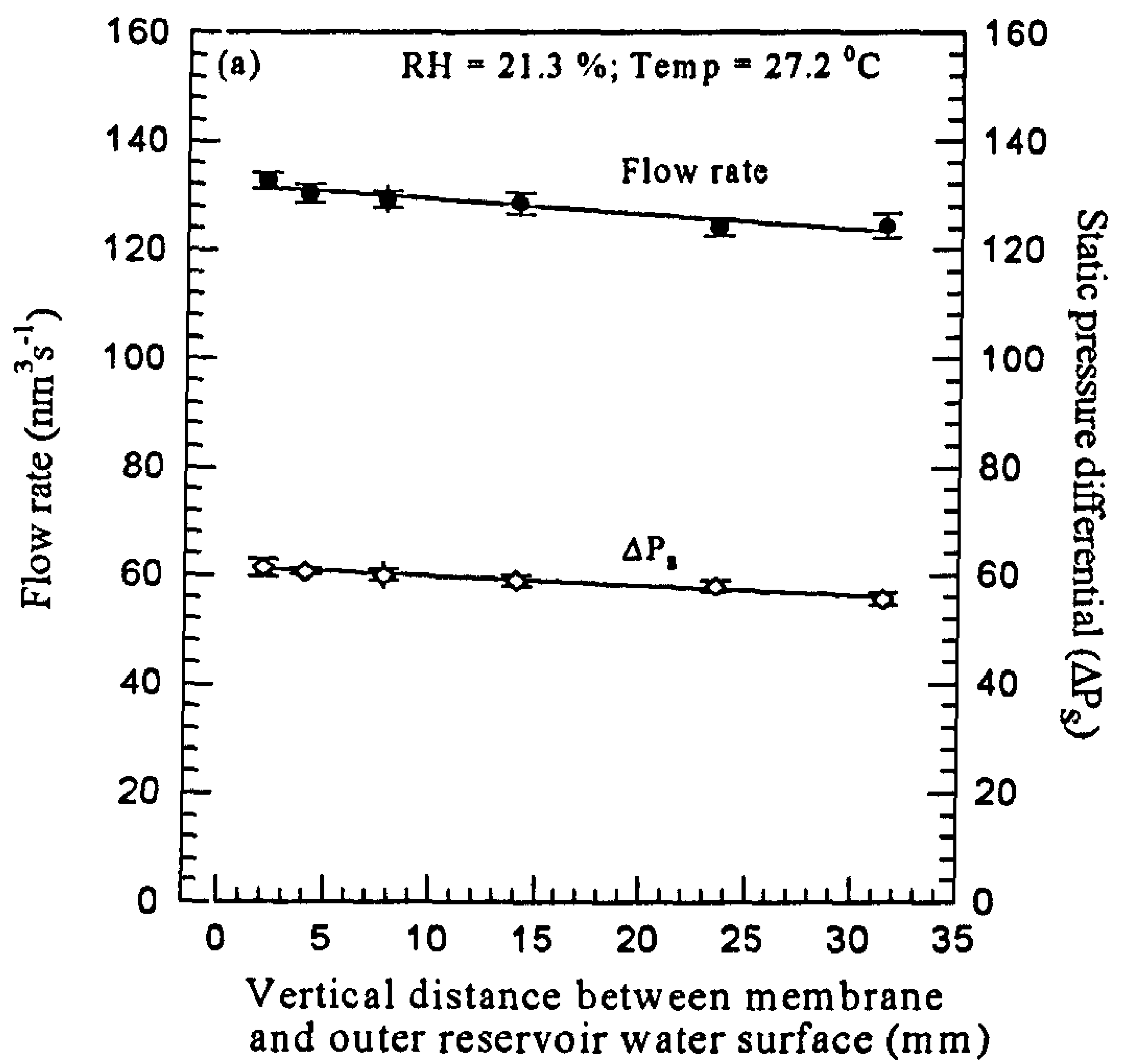



**Fig. 3.04.** Showing the effects on static pressure ($\Delta P_s$) and flow rates of distance between membrane and (a) water level of System IIF (without any Oasis in the inflow turret; MPDi = 0.05 μm, membrane diameter = 50 mm) and (b) water level of System I (MPDi = 0.03 μm, membrane diameter = 25 mm). Each symbol represents a mean ± SE of 5 replicates. Head space depth is the distance between the membrane and free-water surface below.



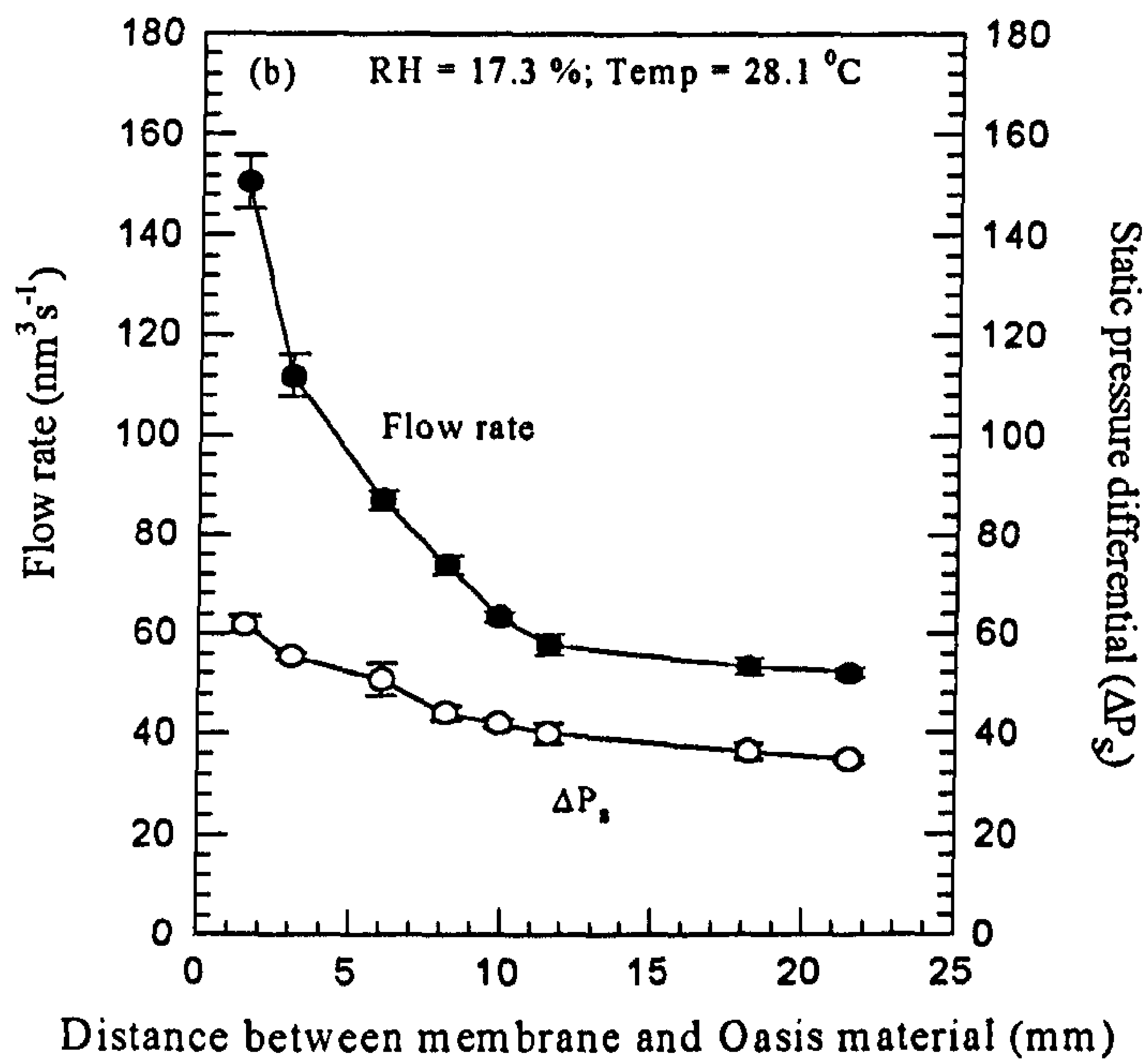




**Fig. 3.05.** Showing the effects on static pressure ($\Delta P_s$) and flow rates of distance between membrane and (a) reservoir water level and (b) level of water saturated Oasis of System IIF (MPDi = 0.05 µm, membrane diameter = 50 mm). Each symbol represents a mean ± SE of 5 replicates. (Note that in system IIF the free water surface depends on the capillary action of the oasis material).

Similarly, when the distance between inflow membrane and water saturated Oasis material ($W_o$) was increased, the static pressure and flow rate gradually decreased (Fig 3.05b). Highest flow rate and static pressure values were recorded when $W_o$ = 1.5 mm (*ca.* 150 $nm^3s^{-1}$ and 62 Pa respectively).

For both Systems I and II, the nearer the inflow water surface, was to the membrane, the steeper was the diffusion gradient and hence the greater the tendency for the inward diffusion of dry gases from the atmosphere. Therefore, pressurisation and HIC flow rate increased simultaneously with decreasing values of water levels.

It should be noted that when Oasis material was used in the inflow turret (System II), the distance between the membrane and the water surface ($W_r$) of the turret *reservoir* has only a small influence on the magnitude of $\Delta Ps$ and HIC flow rates (Fig. 3.05a). The Oasis material in the inflow turret (below the membrane) was always "saturated" with water and the distance between membrane and the surface of the water-saturated oasis material was $\leq$ 2.0 mm, and this is the most important factor for developing a steep diffusion gradient across the membrane. The highest $\Delta P_s$ and HIC flow rates in this instance were *ca.* 61 Pa and 133 $nm^3$ $s^{-1}$ respectively, and this corresponded with a distance, $W_r$, between the membrane and the reservoir water level of 2 mm. On the other hand when $W_r$ was 31.5 mm, $\Delta P_s$ and HIC flow rates were 56 Pa and 124 $nm^3$ $s^{-1}$ respectively. Therefore, the influence of reservoir water level on $\Delta P_s$ and HIC flow rates is very little. The slight decreased in $\Delta P_s$ and HIC with increase in $W_r$ was probably due to the loss of water potential in the capillaries of the Oasis as the 'free water' surface fell.

Various of the equations in Section 2.6 can help to explain the observations of these experiments. The diffusion rate ($m^3$ $s^{-1}$), $J_{wv}$, for water vapour loss from the free water surface within the inflow turret (Fig. 3.06) can be calculated by using equation 2.01: viz. $J_{wv} = (P_{swv}/P_a) / (R_h + R_{md} + R_b)$, and following normal convention (Armstrong

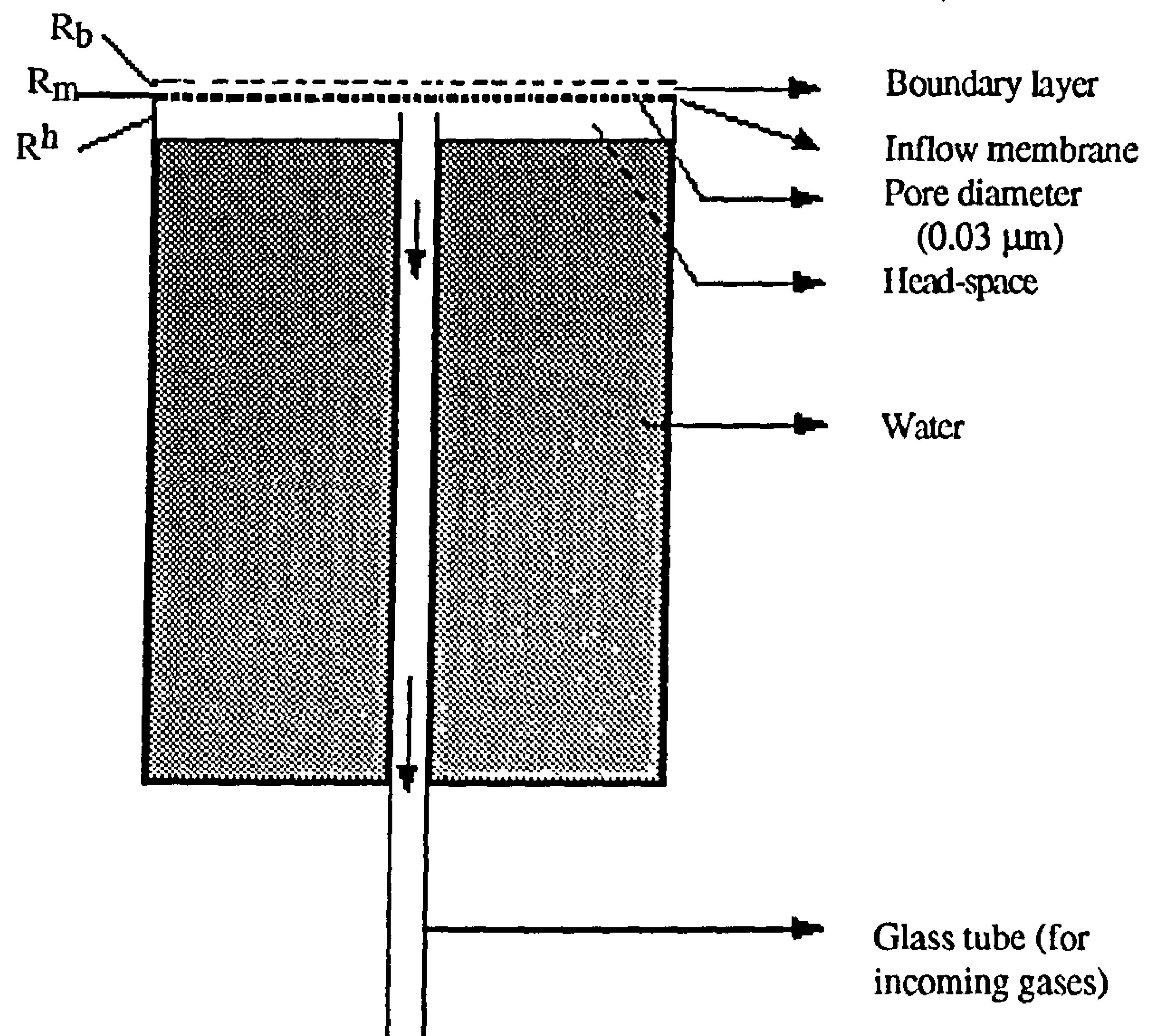



**Fig. 3.06. Inflow turret of through-flow ventilation apparatus (System I); $R_b$ = diffusive resistance with boundary layer; $R_m$ = diffusive resistance of membrane (pore space); $R_h$ = diffusive resistance of head-space.**

1979), the diffusive resistance (s $m^{-3}$) of the inflow turret head-space can be determined from following equation.

$$R_h = L_h / D_o A_x \qquad (3.01)$$

Where, $L_h$ = the distance (m) between the water surface and the membrane, $D_o$ = the mutual diffusivity ($m^2$ $s^{-1}$) of water vapour and air, $A_x$ = cross sectional area ($m^2$) of the head-space (equal to the membrane surface area).

It follows from this equation that when the value of L is decreased (by raising the evaporating water surface of the inflow turret) the value of $R_h$ is reduced. Also, according to the equation (2.01) a decrease in $R_h$ means an increase of $J_{wv}$ value and the diffusion rate will be higher when the distance between the evaporating water surface and Nuclepore membrane becomes lower (if $R_b$, $R_m$, and $\Delta C$ are considered as constant) Both $\Delta Ps$ and HIC flow rate should increase with a decrease in $R_h$ and hence the experimental result for System I accorded with this mathematical analysis. In case of System II, the results may at first seem not to correspond with this mathematical analysis but actually in this system $\Delta P_s$ and HIC flow rate were not controlled by reservoir water level, but by the evaporating surface of the water-saturated Oasis material. The function of reservoir-water is only to make the Oasis water saturated.

### 3.4.2. Within batch variation of inflow and outflow membrane

The aim of this experiment was to find out whether membranes were reliable for inducing constant rates of inflow and outflow.

There was very little variation in HIC flow rate and $\Delta P_d$ for different combinations of inflow and outflow membrane turret samples: standard errors were only ± 0.55 and ± 1.95 % respectively (Table 3.01). It was concluded that such in-batch variation was not sufficient to be of any concern.

**Table 3.01.** Estimation of the ranges of humidity induced convective flow rates and dynamic pressure ($\Delta P_d$) values produced by different turrets of System I; membrane diameter = 25 mm; distance between inflow membrane and water surface W = 2 mm; average ambient T = 19.6 $^0$C and RH = 38.5%.

| Inflow turret = A MPD = 0.03 µm | Outflow turret = B MPD = 0.2 µm | Dynamic pressure (Pa) | Flow rate ($nm^3s^{-1}$) |
|---|---|---|---|
| A1 | B1 | 11.0 | 9.82 |
| A1 | B2 | 10.1 | 9.49 |
| A1 | B3 | 10.1 | 9.49 |
| A1 | B4 | 12.5 | 9 16 |
| A2 | B1 | 10.6 | 9.16 |
| A2 | B2 | 11.0 | 9.49 |
| A2 | B3 | 09.5 | 9.49 |
| A2 | B4 | 09.1 | 9.16 |
| A3 | B1 | 09.6 | 9.33 |
| A3 | B2 | 11.1 | 9.49 |
| A3 | B3 | 10.1 | 9.49 |
| A3 | B4 | 11.2 | 9.16 |
| A4 | B1 | 11.0 | 9.49 |
| A4 | B2 | 10.5 | 9.82 |
| A4 | B3 | 10.5 | 9.33 |
| A4 | B4 | 11.1 | 9.49 |

### 3.4.3: Effects of presence or absence of agar or plantlets

This experiment was performed to see if there were any significant differences in the ventilation rates produced in the culture vessels due to the presence or absence of agar and/or plantlets. When the culture vessels were empty, flow rates (System IIF and including outflow membrane) were 80 $nm^3 s^{-1}$ (Fig. 3.07), and neither the presence of agar (10 ml) in the culture vessels or plantlets significantly altered the flow.

The results confirm that the apparatus in its complete form (inflow + outflow membranes in position) performs as anticipated and is unlikely to be influenced by the activity of the propagated material.

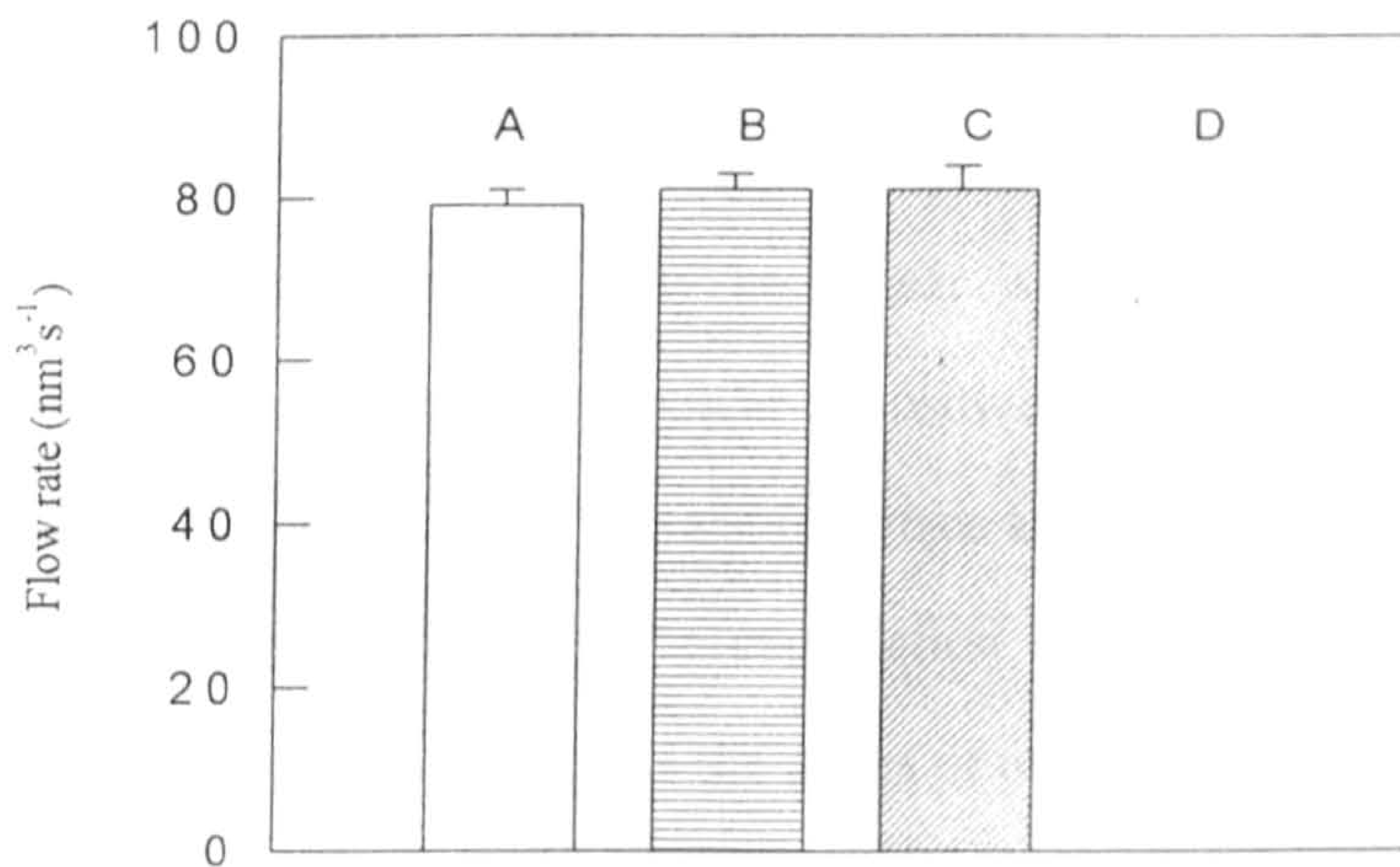



**Fig. 3.07.** Showing the flow rates of ventilation system IIF : A) empty culture vessels, B) culture vessels with 10 $cm^3$ agar, C) containing a tobacco plantlet + agar and D) containing a tobacco plantlet + agar, but with no water in the apparatus (non through flow condition); MPDi = 0.05 µm and MPDo = 0.2 µm; distance between membrane and water saturated oasis (Wo) in A, B and C was 1 - 2 mm from inflow membrane. Ambient temperature was 25°C and RH was 30%. Each symbol represents a mean ± SE of 5 replicates.

### 3.4.4. Effects of membrane area

**Inflow turret**

Flow rates decreased substantially as the area of exposed membrane of the inflow turrets decreased. Static pressures, on the other hand, increased but by relatively small amounts (Fig 3.08). The latter is thought to have been due chiefly to the decreased escape of water vapour overall through the smaller areas of membrane, leading to higher concentrations of water vapour below the membrane. Thus, a decrease in the membrane area to head-space area ratio could be one way of increasing the humidity of the gas-flow delivered by this type of apparatus.

The decrease in flow with decreased membrane area appears to be linear for each of the Systems, and as has already been mentioned, if membrane area and turret area are equal and decrease together in the same proportions (Section 2.7.4), flow rate should be directly proportional to membrane area. In the present examples, however, it can be seen that although the decreases in flow are linear with decreasing membrane areas, the extrapolated lines will meet the y-axis at a positive flow. Clearly there can be no flows with zero membrane areas, but the question arises as to why the flows do not fall linearly to zero at zero membrane area. The explanation is probably the same one offered to explain the rises in static pressure. As the ratios of membrane areas to turret areas decline, the increasing water vapour concentrations in the inflow turret head-spaces will increase the concentration gradients for gas entry and stimulate greater diffusive inflows per unit areas of membranes.

**Outflow turret**

It will be seen that the area of the outflow membrane can also significantly alter the flow rates (Fig. 3.08d): flow rates increased substantially with increasing outflow membrane area. However, the rise can be seen to be curvilinear and is approaching an asymptote at the membrane area normally used for the fast flow apparatus (*ca.* 17 $cm^2$). Thus, in its

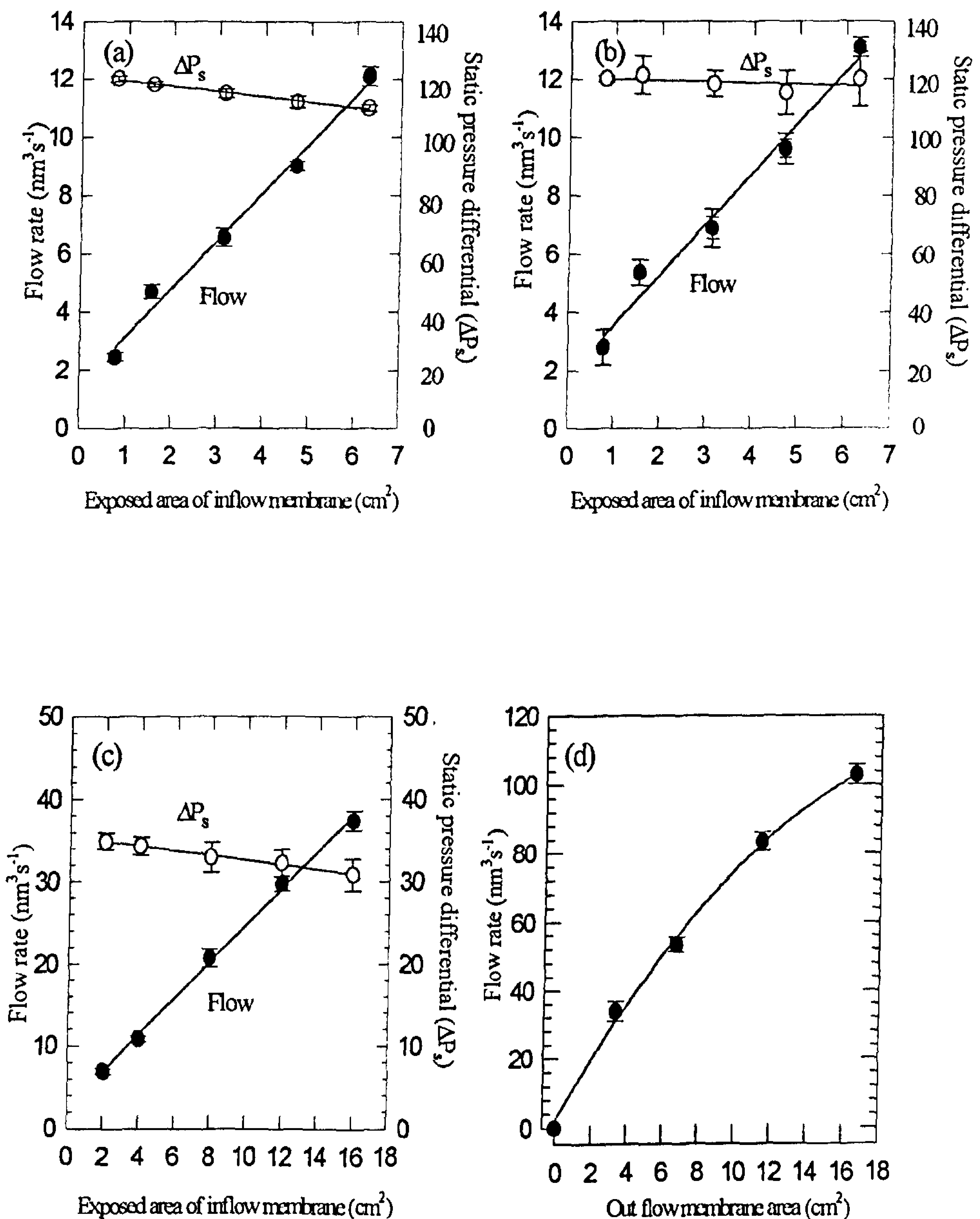



**Fig. 3.08.** Showing the effects of exposed membrane area on the rate of convective flow and the development of static pressure using (a) System I (MPDi = 0.03 μm; W = 3.5 mm), (b) System IIS (slow flow); MPDi = 0.03 μm; Wo = 1.5 mm; and (c) System IIF (fast flow); MPDi = 0.05 μm; Wo = 1.5 mm; (d) effect of outflow membrane area - System IIF (fast flow); MPDi = 0.05 μm; Wo = 1.0 mm. Ambient temperature and RH for (a), (b) and (c) was 19°C and 41%; for (d) 24°C and 21%. Each symbol represents a mean ± SE of 5 replicates. In each case MPDo = 0.2 μm.

normal configuration, the flow rates with System IIF will not normally be much reduced by the presence of the outflow membrane.

The effect of the outflow membrane in System I and System IIS was explored also (data not shown) and it was found that the resistance imposed by the presence of the outflow membrane does not greatly reduce the flow.

### 3.4.5. Effects of pore diameter

Convective flow rates increased with increasing pore sizes rising to a peak at 0.2 μm MPD (Fig. 3.09) and then declined again with further increase in pore size. This confirms experimentally the predictions reported by Armstrong 1992 and by Armstrong and Armstrong (1994b) and the implications have already been discussed at length in Section 2.7.2.

### 3.4.6. Effects of varying dry-air wind speed across the membrane

Wind speed over the inflow membrane has a great effect on static pressure values and also on HIC flow rates (Armstrong 1992; Fig 3.10). With increasing wind speed both static pressure and HIC flow rates increased, the rises being particularly steep up to 0.01 m $s^{-1}$. At higher wind speeds the plots began to plateau and above 0.03 m $s^{-1}$ the slope became very shallow. This can be explained in terms of boundary layer thicknesses: the faster the dry air wind speed, the thinner will be the boundary layer, and the drier will be the air near to the membrane. This will steepen the humidity gradient across the membrane and simultaneously, the faster will be the flow rate and also higher the static pressure value. The results suggest that the apparatus will work best in conditions where wind speeds exceed 0.03 m $s^{-1}$, and since wind flow in the growth room usually varied between 0.1 to 0.3 m $s^{-1}$ it may be deduced that wind flow conditions in the growth room were close to optimum for the functioning of the apparatus.

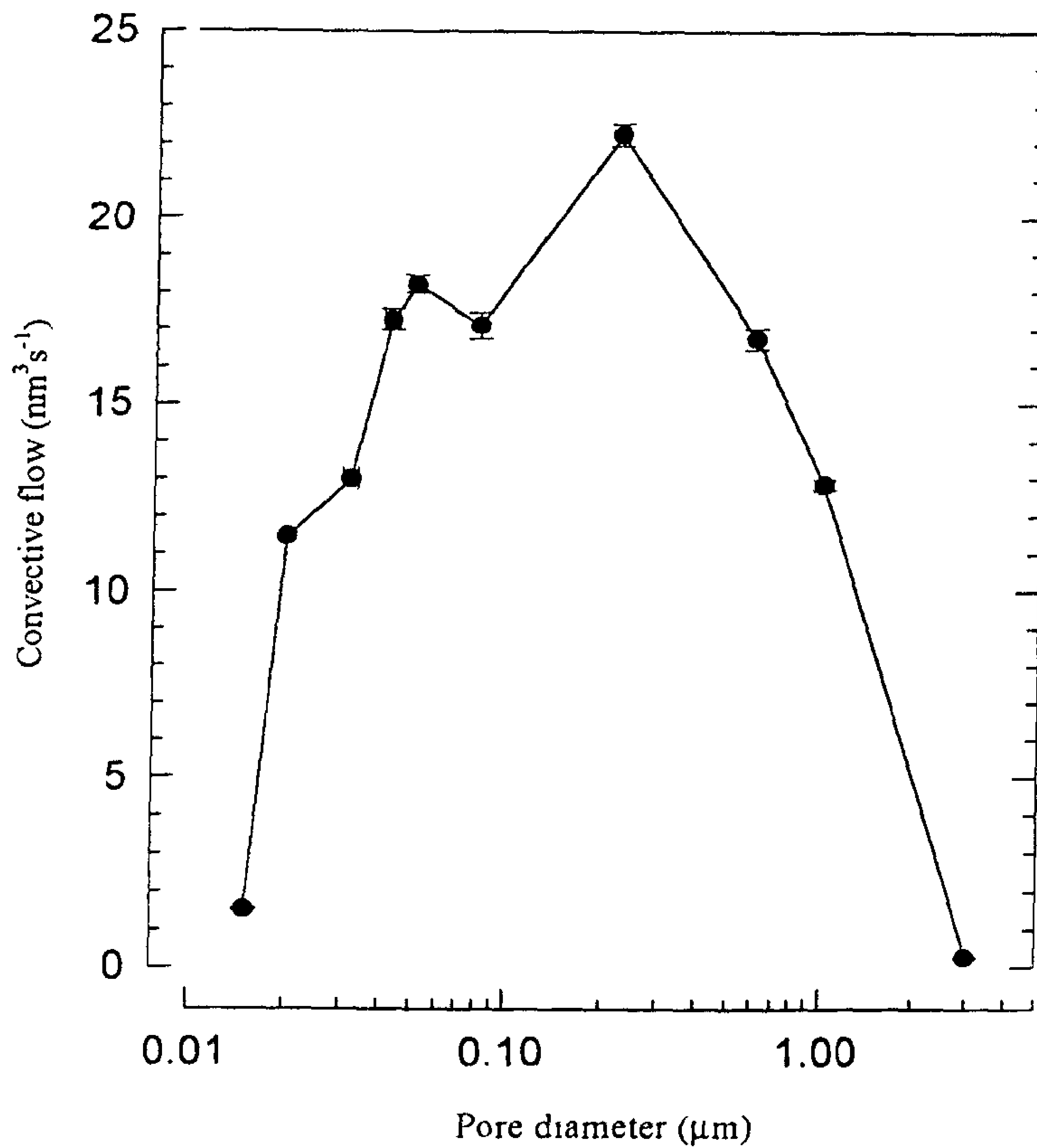



**Fig. 3.09.** The relationship between convective flow rate and pore diameter of the inflow membrane of System I (membrane diameter = 25 mm, W = 4 mm). The wind speed of dry air across the membrane was 0.044 $ms^{-1}$. The ambient temperature was 19.4°C and the RH was 39.4%. Each symbol represents a mean ± SE of 5 replicates.

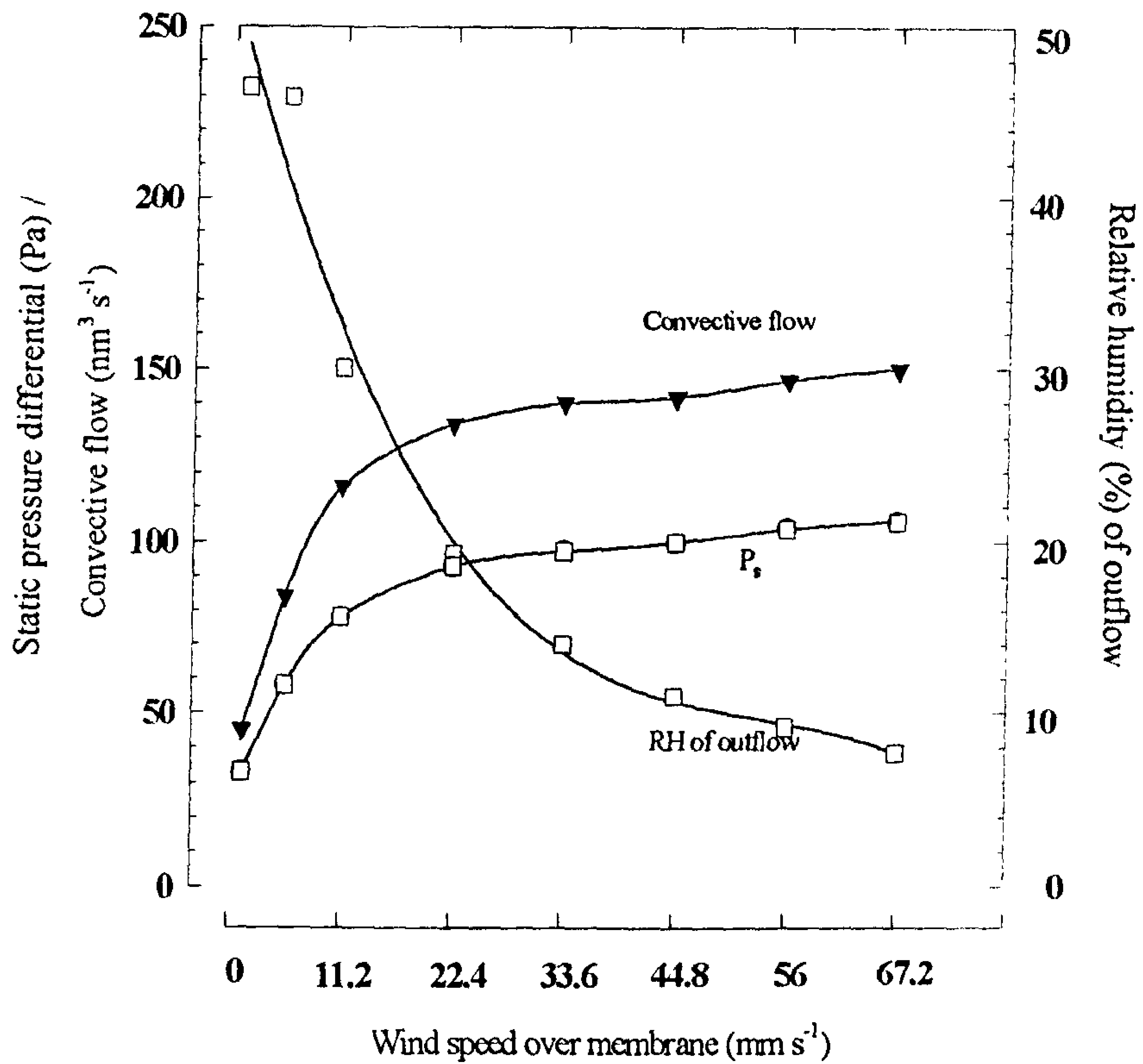



**Fig. 3.10.** The effects of wind speed of dry air (RH = *ca.* 0%) on $\Delta P_s$ , HI-convection and humidity of air stream after passing over the membrane; MPDi = 0.046 µm; W = 4 mm. (After Armstrong 1992).

### 3.4.7. Effects of varying the humidity of wind across the membrane

Static pressures and HIC flow rates were greatly reduced with increasing relative humidity (RH) in the external atmosphere above the membrane. At 0% external RH the static pressure and HIC flow rate were 181 Pa and 7.7 $nm^3 s^{-1}$ respectively, whereas at 54% RH, $\Delta P_S$ was *ca.* 83 Pa and HIC, 3.6 $nm^3 s^{-1}$ (Fig 3 11) At 100% RH, extrapolation of the graphs showed that there should be virtually no pressurisation or flow, because of no humidity gradient and hence no concentration gradient for the inflow of atmospheric gases (Fig 3.12). Conversely, the drier the outer air, the steeper will be the diffusion gradient; as a result pressurisation and HIC flow rates increase. These results are in agreement with Armstrong (1992) and Armstrong and Armstrong (1990, 1994b). It is interesting to note that the relationships between flow and RH and static pressure and RH are linear and thus the effects of humidity changes in the external atmosphere are relatively easily predicted. However, it must be borne in mind that the higher the ambient temperature the steeper the slopes.

The implications of these data are that it is most desirable to have the apparatus situated in conditions of low humidity. Relative humidities in the growth room during the present study varied from a little under 20% to *ca.* 40%

### 3.4.8. Effects of increasing the resistance to venting

#### 3.4.8.1. Outflow membrane resistance: System I.

As the resistance to venting was increased stepwise by lowering the pore diameters of the outflow membrane the flow rate decreased (Fig 3.12c). Over the pore diameter range 0.4 to 0 1 μm the decline was only small but below 0.1 μm the fall was dramatic. Conversely, but as expected, dynamic pressures rose with declining pore diameter and again in the range 0.4 to 0.1 μm the change was only small, and below 0.1 μm there was a steep rise; at MPD 0.03 μm the dynamic pressure reached 55 Pa.

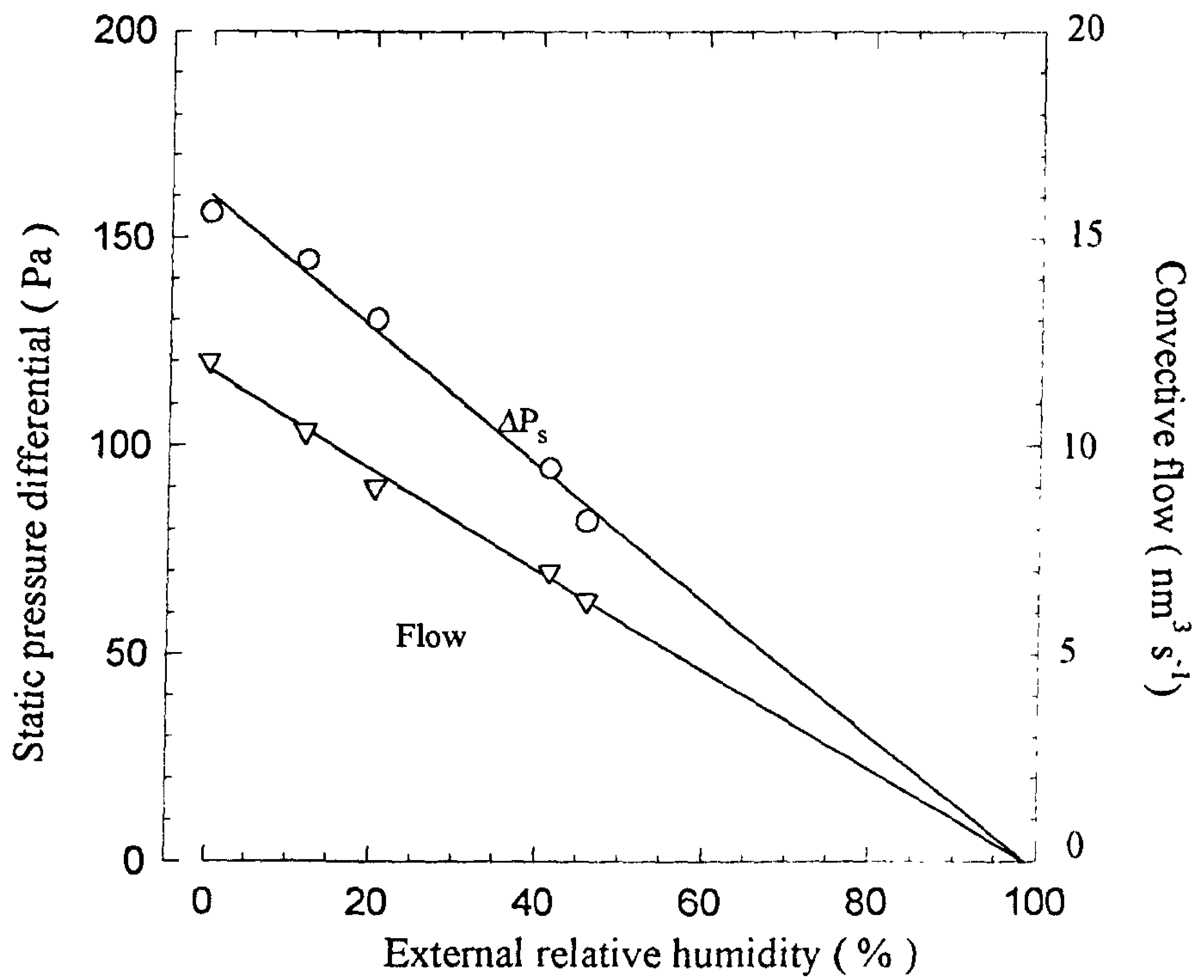



**Fig. 3.11.** Showing the effects of humidity differential on static pressure ($\Delta P_s$) and humidity-induced convective flow rates using System I (MPDi = 0.03 μm, membrane diameter = 25 mm, W = 6.0 mm). Ambient temperature was 19°C and RH was 36%. Each symbol represents a mean of 5 replicates.

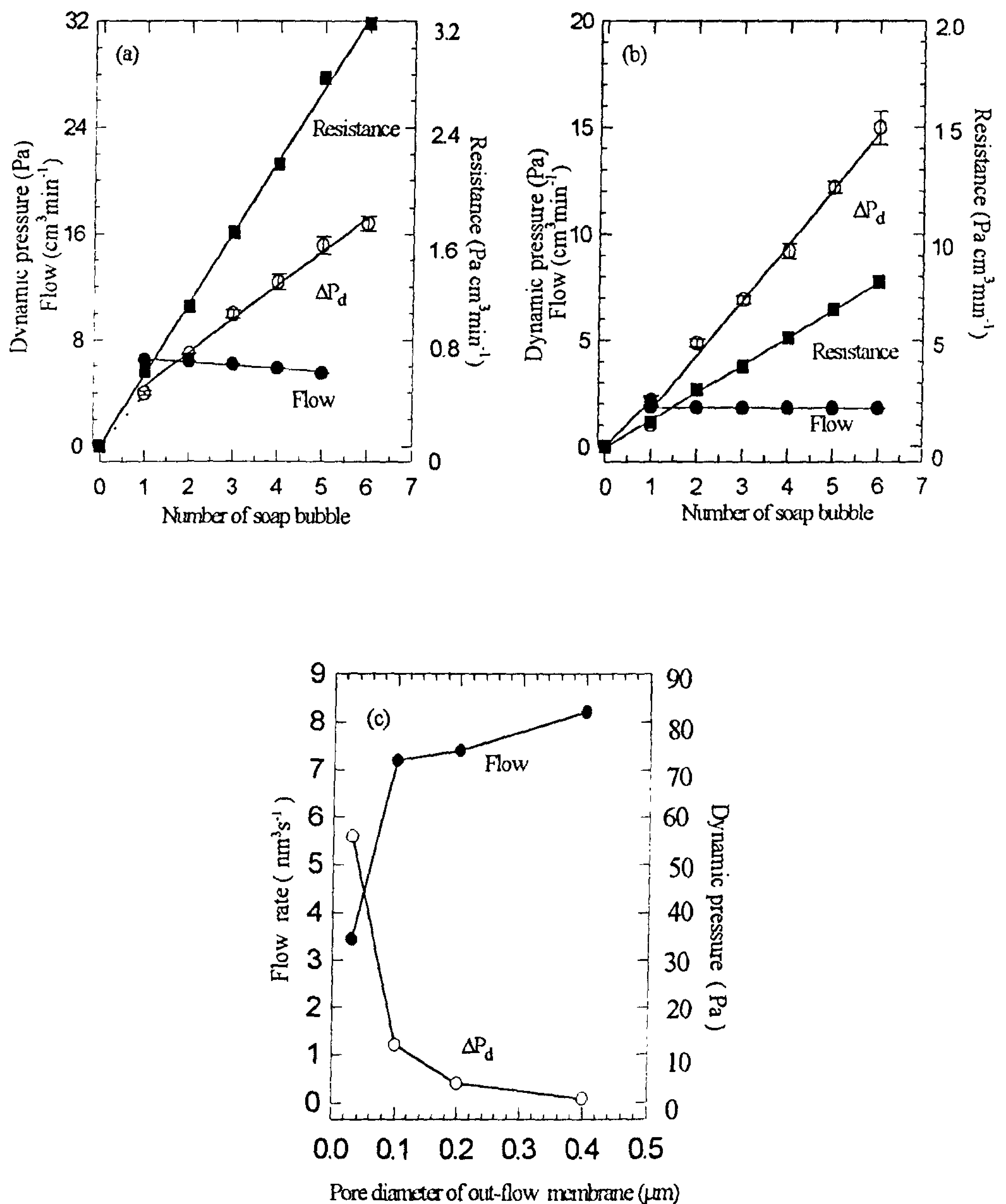



**Fig. 3.12.** Showing the effects of altering the resistance to venting, *either* by placing soap bubbles in series (0,1,2,3,4,5,6) within the flow meter of (a) System IIF (MPDi = 0.05 µm, membrane diameter = 50 mm, Wo = 1.0 - 1.5 mm) and (b) System IIS (MPDi = 0.03 µm, membrane diameter = 25 mm, Wo = 1.0 - 1.5 mm), *or* by using different outflow membranes (c) in System I; MPDi = 0.03 µm; MPDo = 0.03, 0.1, 0.2, 0.4 µm. Ambient temperature was 25°C and RH was 18%. Each symbol represents a mean ± SE of 5 replicates.

These effects clearly demonstrate the changes in the attributes of the membranes when pore diameter falls into the Knudsen regime (section 2.6 and Armstrong, Armstrong and Beckett 1996a,b). At a pore diameter of 0.03 μm there is probably no pressurised flow through the membrane, hence the steep rise in dynamic pressure and increased gas density within the vessel. Thus, gas escape is probably due entirely to Knudsen diffusion from the outflow membrane; in volume terms the concentration gradient driving the outflow will be 55/101300 $m^3$ $m^{-3}$ i.e $\Delta P_d$ / $P_a$. At a pore diameter of 0.1 μm, outflow may again be chiefly diffusive but the diffusivity of the membrane will be very much greater, while at 0.2 μm there will be significant pressure flow through the membrane. At 0.4 μm most of the outflow will probably be pressurised bulk flow (see also Armstrong, Armstrong and Beckett 1996a,b).

It should be noted that although flows are increased further when using an outflow membrane of pore diameter 0.4 μm this is not possible for tissue culture. Instead an outflow membrane of pore diameter 0.2 μm must be used to prevent the entry of bacteria and fungal spores into the System. Viruses, however will not be excluded.

### 3.4.8.2. Other venting path resistances

Venting resistance can be increased, and hence dynamic pressure raised and flows reduced, by the soap films used in the soap film flow meters (Fig 3.12 a,b). However, it would seem that flows are not very much reduced by a single film which is the usually the norm when using such devices.

More dramatically $\Delta P_d$ can be increased by stepwise addition of 1 μl Microcap capillaries to the venting path (Fig 3.13). The result agrees with Armstrong (1992) and Armstrong and Armstrong (1994b) and demonstrates the importance of minimising the resistance of any pipe-work within this type of apparatus.

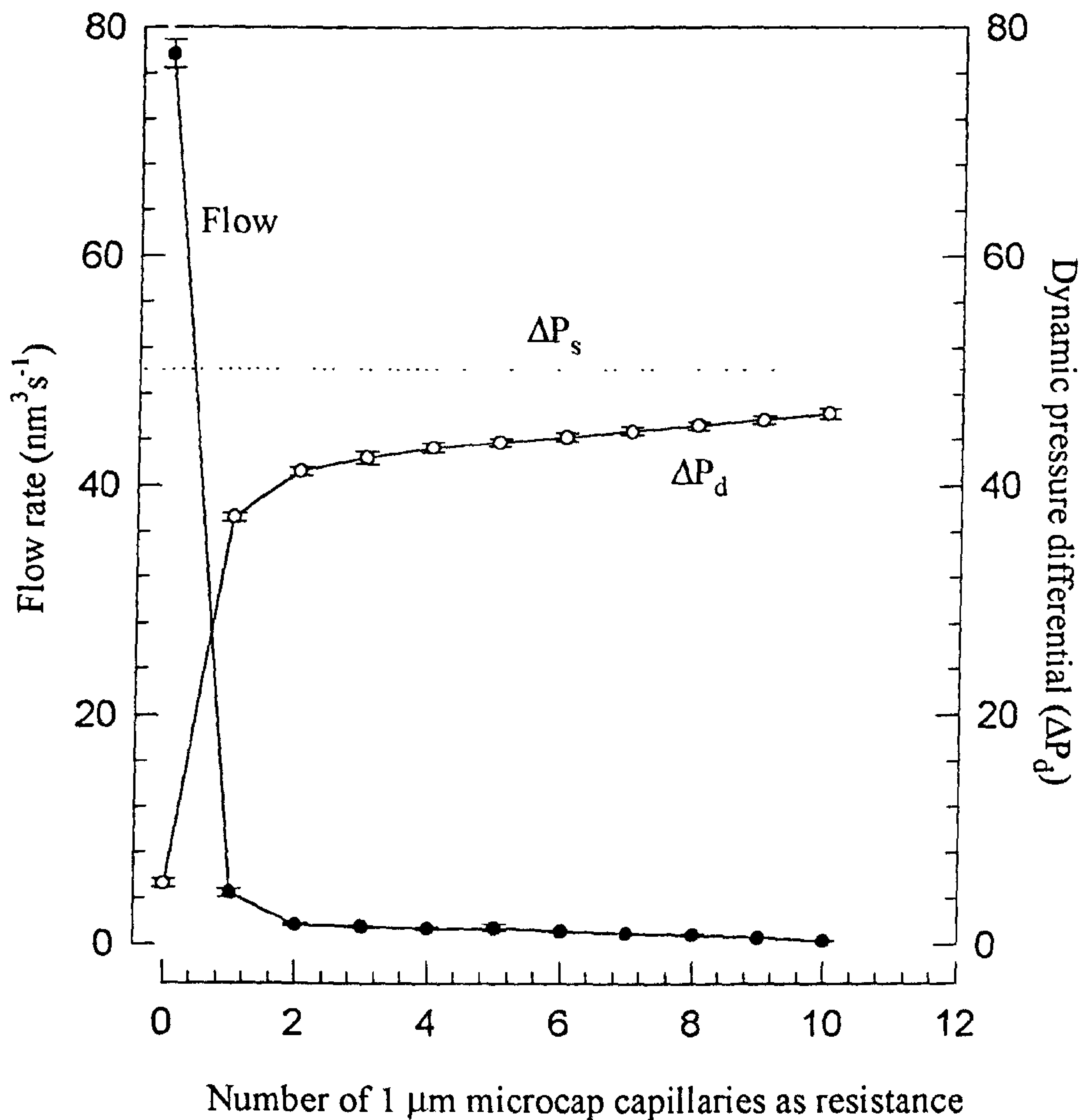



**Fig. 3.13.** Showing the effects of increasing the resistance to venting of System IIF on the development of dynamic pressure ($\Delta P_d$) and the flow rates. Note that the static pressure ($\Delta P_s$) for this membrane under these conditions was 51 Pa. Ambient temperature was 25°C and RH was 20%. Each symbol represents a mean ± SE of 5 replicates. MPDi = 0.05 μm, membrane diameter = 50 mm, Wo = 1.0 - 1.5 mm.

### 3.4.9. Sustainability of flow rates

The aim of this experiment was to determine the ability of the forced ventilation systems to sustain the ventilation of the culture vessels without renewing with water.

Using System I (Fig 3.14 b,d) the water level dropped each day, and simultaneously the flow rates decreased rapidly: even over the first day the flow fell by 25% and by day four the rate was only 30% of that at the start; this latter value was only 8% of the flow rate in System IIF after four days. After *six* days without attention, the flow rate from System IIF was *ca.* 70 $nm^3 s^{-1}$, compared to only 4 $nm^3 s^{-1}$ from System I: *i.e.* System II at this stage is > 17X more efficient Thus if a relatively constant flow rate is to be maintained with System I, the water level of the inflow turret should be adjusted daily to keep it as near to the membrane as possible.

The results in Fig 3.14 a and b show that although the water level of the water reservoir of ventilation System IIF dropped each day, flow rates remained almost steady for up to 4 days. This is the result of the capillary action in the Oasis material. Even after 6 days the flows were still 70% of maximum. It is evident, therefore, that the new apparatus does not require such frequent attention and is much more efficient than System I. It can also be deduced that maximum flow rates could be further prolonged by a relatively small increase in the radial dimensions of the reservoir. For example, increasing the reservoir radius by only 5 mm would double the period of maximum flow.

### 3.4.10. Ventilation system and culture vessel humidity

The relative humidity in an empty culture vessel (60 $cm^3$) receiving convective gas flow from the fast-flow ventilation apparatus (System IIF - flow rate 5.0 $cm^3 min^{-1}$) was found to be *ca.* 65-70% (Fig. 3.15).

Very high relative humidities ($98.3 \pm 1.2$) were recorded in sealed vessels containing agar plus plantlets (Tobacco: F.W. *ca.* 210 mg). When the vessels were capped with polypropylene disc slightly lower humidities ($93.2 \pm 1.1$) were achieved. However, when

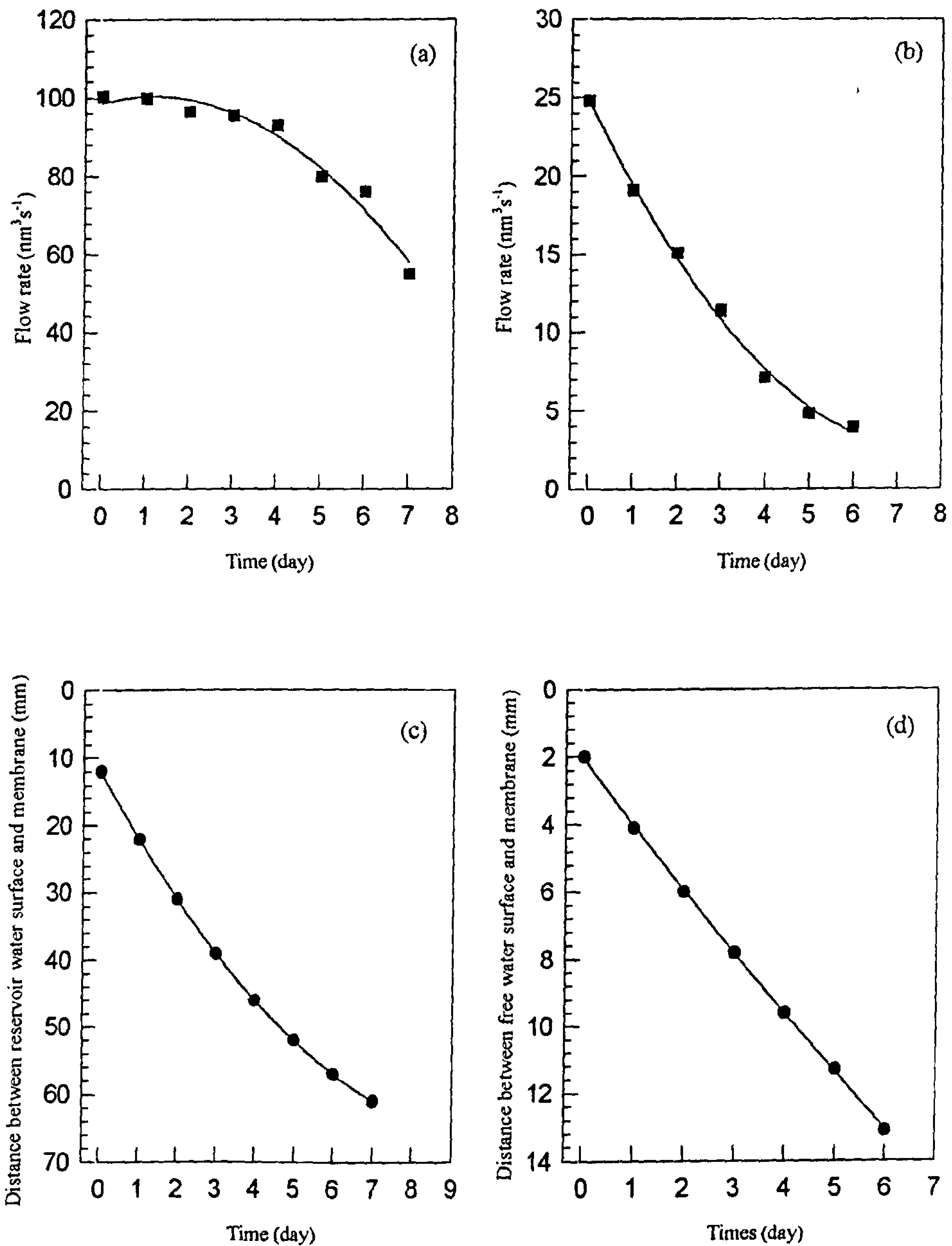



**Fig. 3.14.** **(a) & (b):** The sustainability of the flow rate without adding water to the systems, i.e. allowing the water levels to drop : (a) System IIF (MPDi = 0.05 μm, membrane diameter = 50 mm, Wo = 1.0 - 1.5 mm) and (b) System I (MPDi = 0.03 μm, membrane diameter = 25 mm).
**(c) & (d):** The effects of time on water depths within (c) the water reservoir of System IIF and (d) the inflow turret of System I. The ambient temperature was 25°C and the RH was 21%. Each symbol represents a mean of 5 replicates (Note that in system IIF the free water surface depend on the capillary action of the oasis material).

capped with the forced ventilation apparatus the RH fell to 85.2±0.9 % in the vessels containing agar + plantlets; in the absence of plantlets, (agar only), the RH was *ca.* 80%. Although very significantly higher than in the empty vessel, and due no doubt to (i) the transpiration and respiratory activity of the plantlets and (ii) the evaporation from the agar in the culture vessels, this is a very useful value in terms of plant performance. The literature shows that successful weaning of plants is much more likely if plants can be grown at humidities <94% (Smith *et al.*, 1992). The humidity levels achieved in the culture vessels is also dealt with in Chapter VI.

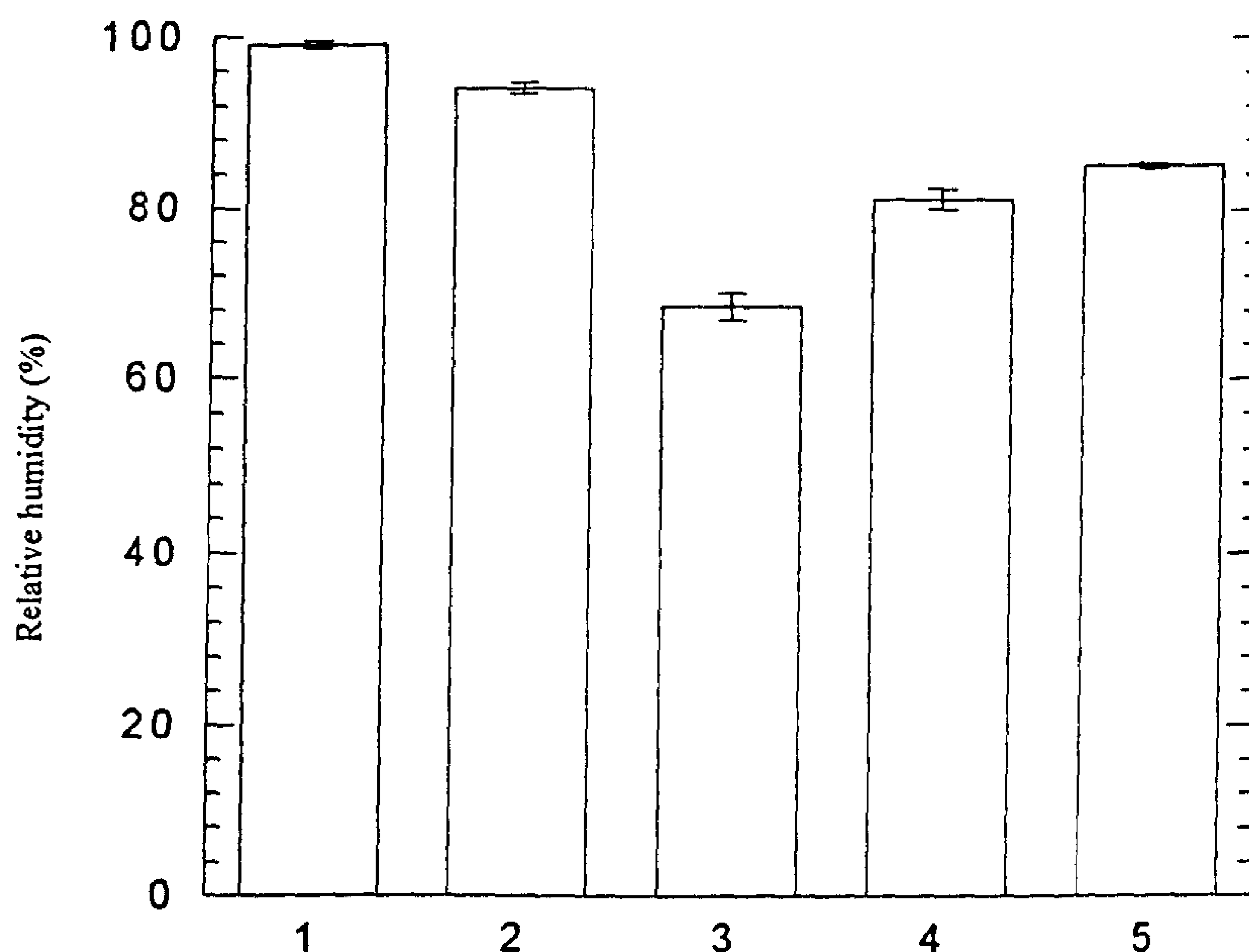



**Fig. 3.15.** Effects of different types of ventilation on % relative humidity of the culture head-space; 1 = each vessel contained agar + plantlet and sealed with silicone rubber bung; 2 = each vessel contained agar + plantlet and capped with polypropylene disc (diffusive ventilation); 3 = each vessel empty (no agar or plantlets) and fitted with a FF-ventilation apparatus; 4 = each vessel contained agar (no plantlet) and fitted with a FF-ventilation apparatus; 5 = each vessel contained agar + plantlet and fitted with a FF-ventilation apparatus. Ambient RH was *ca.* 30% and the temperature was 25$^0$C. Volume of culture vessels = 60 $cm^3$; volume of agar medium = 10 $cm^3$ ; tobacco plantlets were 20 day-old (FW = *ca.* 210 mg); fast flow ventilation rate = 3.5 $cm^3$ $min^{-1}$.

### 3.4.11. Ventilation systems and $t_{50}$'s for retention of injected ethylene

A major reason for developing forced ventilation systems for use in plant micropropagation is to try to prevent, or at least minimise, the build up of gaseous products of metabolism. A principal consideration is the removal of ethylene, a potent plant hormone, which at high concentrations can be a growth inhibitor and a cause of senescence.

The results shown in Fig. 3.16 demonstrate the efficacy of ethylene removal in the three types of microprop ventilation systems tested: completely sealed, diffusively ventilated by capping with a polypropylene membrane and forced ventilation

Clearly forced ventilation was the most effective method for removing ethylene from the culture vessels: the fast-flow system was *ca.* 18 times and the slow-flow one *ca* 4 times more efficient in removing ethylene (and presumably any other accumulated gases) compared with polypropylene membranes - one of the most commonly used diffusive methods for ventilating culture vessels in plant micropropagation. As would be expected, the dry turrets (large and small) had long $t_{50}$ times (227 min and 285 min) since, when dry, they are unable to produce any convective flow and the gas loss can take place only by diffusion.

Ethylene loss from the sealed vessels was very slow indeed· after nearly 800 minutes there was still 80% of the injected sample present. Of the 20% that had been lost, it is possible that most had diffused through the relatively thin-walled Suba-seal on the vessel side-arm, but some may also have diffused out via the silicone rubber bung.

The results suggest that the forced ventilation systems are the most effective for the removal of accumulated ethylene from the culture head-space.

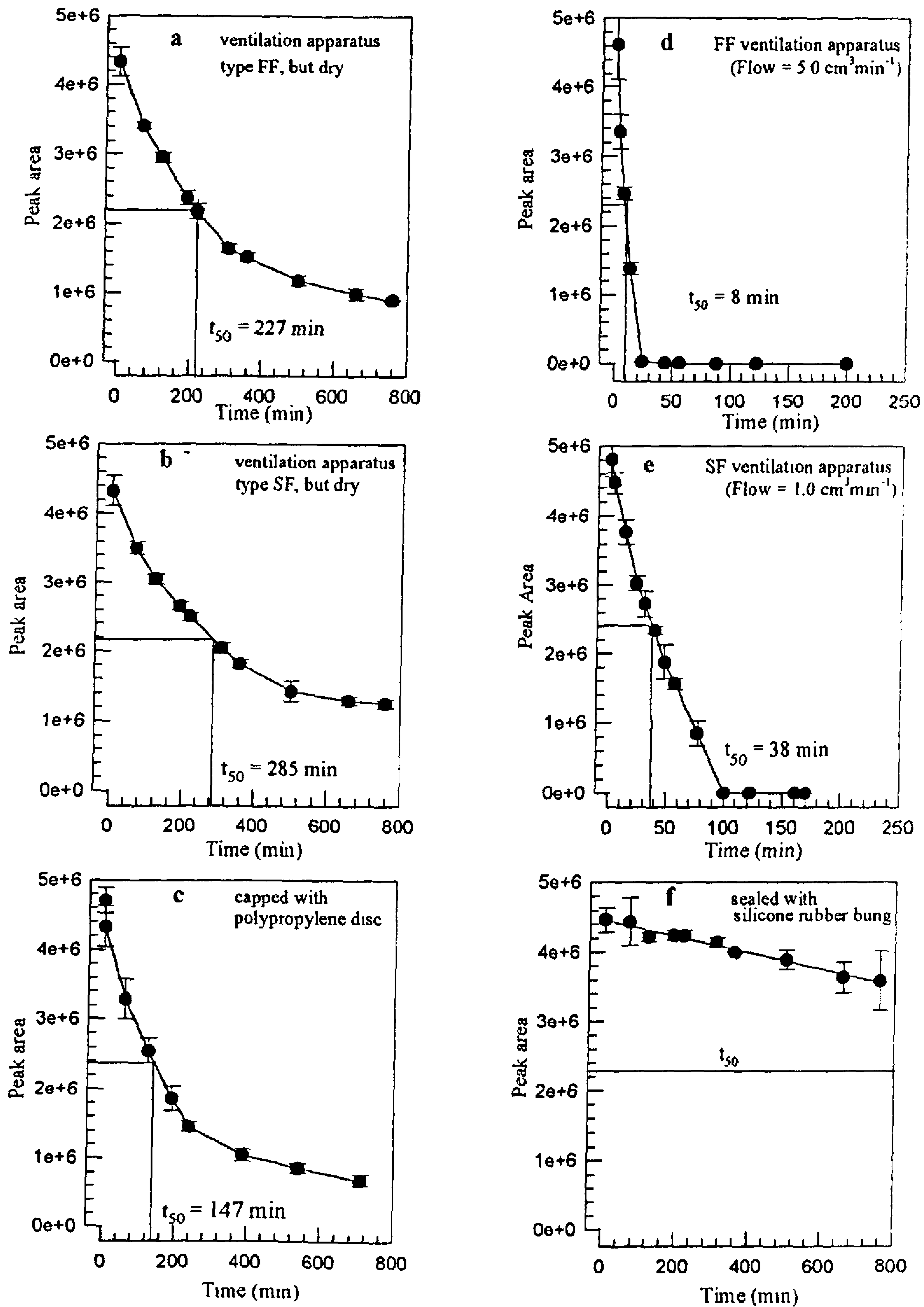



**Fig. 3.16.** The influence of different types of 'capping' of culture vessels on retention times, $t_{50}$, for injected ethylene; initial concentration was 23 µl $l^{-1}$ of ethylene in air.
a, b and c : with diffusive ventilation; d & e : with forced ventilation (System II); f : sealed : $t_{50}$ by extrapolation is > 2000 min. Culture vessel volume was 60 cm$^3$; each symbol represents a mean ± SE of 10 - 4 replicates .

### 3.5. FINAL COMMENTS

This part of the study has highlighted the improvements in the design of System II over System I. By incorporating a water reservoir and using Oasis material in the inflow turret of System II, there is little danger of wetting the membrane, and the maintenance of a steep diffusion gradient is facilitated. This, together with the use of an inflow membrane of larger diameter, has led to considerably faster flows (5 $cm^3$ $min^{-1}$) and more sustained flows (> 5days), without the apparatus requiring attention. (System I must be filled with water daily.) Even after a week the flow from System II was still 2X the maximum from System I. Flows with this new system can be 4X the maximum for System I (Fig. 3.14). Also the new model, being comprised of one unit, is a more convenient capping system for tissue culture vessels.

In addition, the study has shown that membrane pore diameters, external RH, wind speed across the inflow membrane and resistance to venting can all have important effects on the velocities of flows through culture vessels. Clearly, these factors must be considered in future in further modifications to the system for the ventilation of tissue cultures *etc.*

The $t_{50}$ measurements for the escape of ethylene from 60 $cm^3$ vessels have shown that System II used in the normal "throughflow mode" is > 25X more effective than when used as a simple diffuser; it is also > 18X more efficient for removing ethylene than conventional capping by a polypropylene membrane.

Subsequent chapters provide data comparing the performance of the new System II with those of conventional capping systems, in relation to the growth and physiology of various plant species

# CHAPTER IV

## EFFECTS OF CLOSED SYSTEM, DIFFUSIVE AND FORCED VENTILATION ON THE GROWTH AND PHYSIOLOGY OF CAULIFLOWER *IN VITRO*

### 4.1. INTRODUCTION

Growth and development of *in vitro* cultured plantlets or tissues depend not only upon the composition of the nutrient medium but may also be affected by the composition of the gaseous atmosphere (Blazková *et al.* 1989; Jackson *et al.* 1991). The conventional protective conditions under which the tissues are grown to prevent microbial contamination and retard desiccation of the plant and the nutrient medium may often unintentionally cause a restriction of gas exchange between the vessel atmosphere and the outside air (Buddendorf-Joosten and Woltering 1994). For example with polypropylene film over the mouth of the vessel, condensation droplets may develop on the inner surface and impede the diffusive exchange of gases through the pores. Similar effects might develop with cotton wool bungs. However, the complete sealing of culture vessels is often the normal practice and here, of course, there is very little, if any, gaseous exchange with the atmosphere.

Further to this, tissue cultured plants themselves can release a variety of substances into the atmosphere of the culture vessels and these may accumulate under conditions of restricted ventilation and have significant effects on growth and development of *in vitro* cultures (Heyser and Mott 1980). The most widely studied gaseous product from cultures is ethylene (Gamborg and LaRue 1968, Huxter, Reid and Thorpe 1979) and this was first shown by Stewart and Freebairn (1967). The effects of ethylene can be prevented by potent inhibitors of both ethylene action and biosynthesis (Yang 1985) or by forced ventilation (Saltveit and Yang 1987). Other volatile substances released *in vitro* are carbon dioxide (Zobel 1987), ethane, ethanol, methane, acetylene

and acetaldehyde (Thomas and Murashige 1979a,b; Adkins, Shiraishi and McComb 1990). Changes in the gaseous atmosphere of the culture vessel can suppress the growth of callus and promote necrosis in rice (Adkins 1992), and in other species (Table 1.01). Large amounts of carbon dioxide in the culture vessel atmosphere have been measured for many species grown *in vitro* and often high concentrations are found in association with ethylene (Zobel 1987). The effects of $CO_2$ and $O_2$ availability and the presence of ethylene and other volatiles already have been reviewed (Chapter I: General Introduction).

Cauliflower is one variety of the species *Brassica oleracea* L. that also includes cabbage, kale, kohlrabi, brussel sprouts and broccoli, which are important vegetables for human consumption as well as animal feed (Grout 1988). The poor growth and regeneration of cultured cells and tissues of *Brassica* spp. have been attributed to ethylene produced by the cultured plants (Chi *et al.* 1990). By using ethylene inhibitors many authors have shown that accumulated ethylene was one of the causes of recalcitrance in *Brassica* spp. (Chi and Pua 1989; Chi, Pua and Goh 1991; Lentini *et al.* 1988 and Sethi, Basu and Guha-Mukherjee 1990). However, no reports have been published so far relating the possible roles of ethylene to the growth, regeneration and other physiological activities of *in vitro*-grown cauliflower.

The aim of this part of the study was to compare the effects of a closed system (vessel completely sealed), diffusive ventilation system (vessels capped by polypropylene film) and the forced ventilation systems (SF and FF described in Chapters II and III) on cauliflower seedlings/plantlets grown *in vitro*. In particular, a major aim of the study was to see if forced ventilation might improve the growth of cauliflower seedlings/plantlets by flushing out the ethylene. To this end the effects of ethylene inhibitors ($AgNO_3$, $Ag_2S_2O_2$, and $CoCl_2$) and the ethylene precursor (ACC) were investigated. Also the effects of the different methods of ventilation on carbon dioxide, ethylene and oxygen concentrations inside the culture vessels were examined. The growth of the plants was studied in terms

of leaf number, fresh weight and area, stem fresh weight, length and diameter and root number and maximum length. The effects of ventilation and $\pm AgNO_3$ or ACC on leaf chlorophyll contents and rates of photosynthesis were also investigated.

## 4.2. MATERIAL AND METHODS

### 4.2.1. Plant material

Seeds of Cauliflower (*Brassica oleracea* var. botrytis L.) were surface-sterilized by immersing in sodium hypochlorite solution (2% w:v) for 5 min, then rinsed three times with sterile water and sown at a density of four seeds per tube on to full strength MS medium in culture vessels where normally they germinated within 24 hours.

Hypocotyl cuttings (5-7 mm in length) were obtained from 5 days old seedlings. The cotyledons were dissected out and discarded.

### 4.2.2. Establishment of cultures

Sterilized seeds or cuttings were inoculated into culture tubes containing 10 ml of MS medium. These vessels (60 $cm^3$) had a side arm sealed with a silicone rubber 'Suba-seal' to allow ethylene, oxygen or $CO_2$ samples to be added or withdrawn by a hypodermic syringe. Unless otherwise stated cultures were incubated at 23 - 25°C under cool-white fluorescent light (a continuous light flux; PAR = 150 µmol $m^2s^{-1}$).

The vessels (60 $cm^3$) were 'capped' in various ways : (a) by silicone rubber bungs (b) by conventional polypropylene membranes (thickness = 25 µm) (c) by the slow flow (SF) ventilation apparatus (flow rate 1.5 $cm^3$ $min^{-1}$), and (d) by the fast flow (FF) ventilation apparatus (flow rate 3.5 $cm^3$ $min^{-1}$)

### 4.2.3. Media preparation

MS (Murashige and Skoog 1962) basal medium was used for the culture of plantlets/seedlings under each of the different treatments. As different media constituents were required in different concentrations, separate stock solutions for the macronutrients, micronutrients, Fe-EDTA (Iron stock), vitamins and aminoacids, growth regulators etc. were prepared for ready use (Appendix I). For mixing the solutions a magnetic stirrer (Gallenkamp) was used. The pH of the medium was adjusted to 5.8 by the dropwise addition of 0.1M HCl or 0.1M NaOH, whichever was necessary, and

gelling was achieved by addition of an appropriate quantity of Phytagel (Sigma)(3.0g $l^{-1}$). The culture vessels were then autoclaved at 15 psi pressure at 121°C for 15 minutes.

### 4.2.4. Methods of measuring ethylene, carbon dioxide, and oxygen concentrations

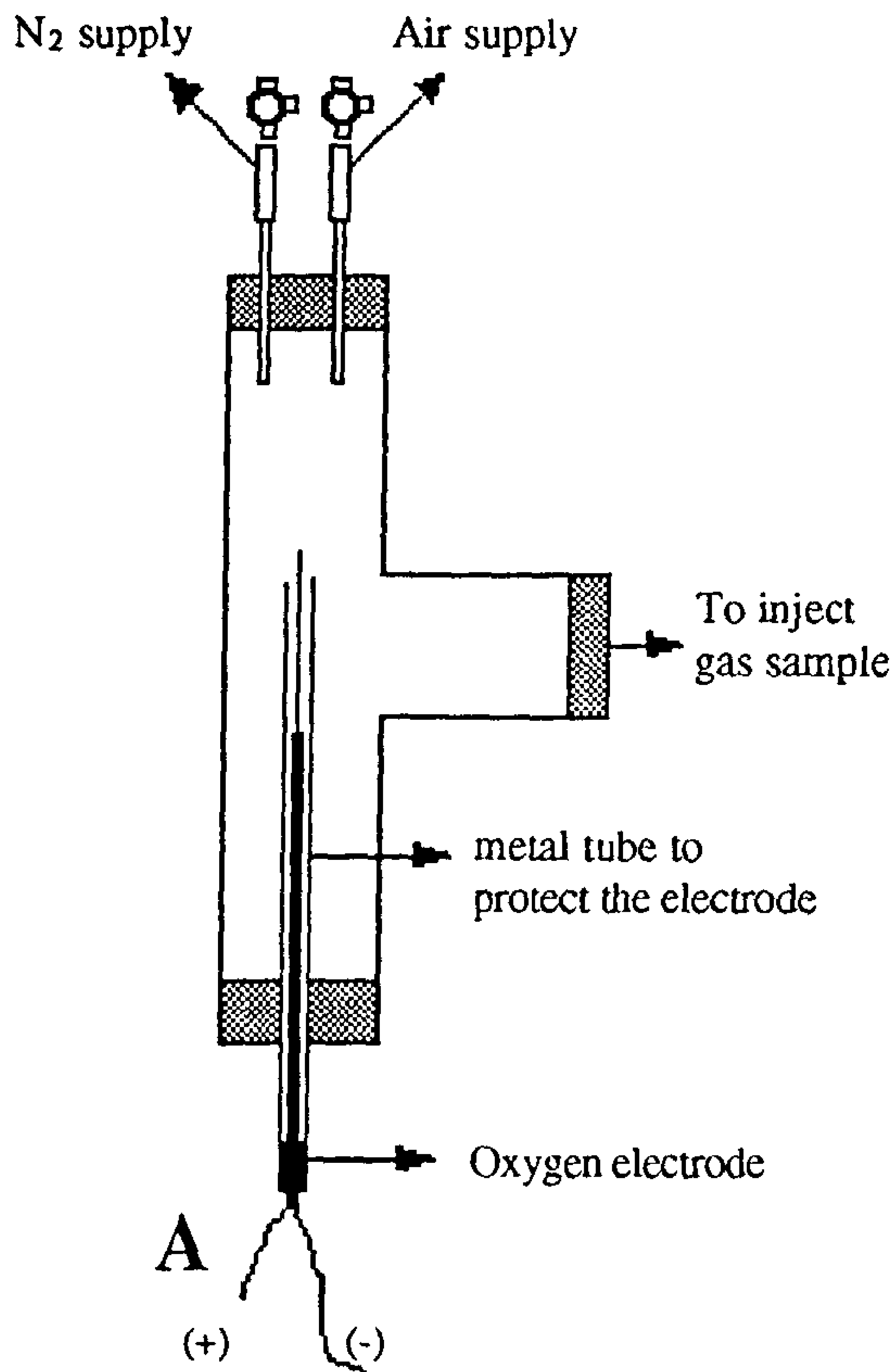
#### 4.2.4.1. Ethylene

For each experiment, when plants were ready for harvesting, the ethylene concentrations were determined by removing 500 µl samples of gas from the culture vessels and analysing by means of gas chromatography (PYE Unicam). Poropack Q (60-80 mesh) was used in a glass column (2500 mm X 6.5 mm) and temperatures of the column, injector and flame ionisation detector were 100, 150, and 150°C respectively. The ethylene peaks were identified by a retention time of about 1.4 min. Nitrogen was used as the carrier gas at a rate of 60 $cm^3$ $min^{-1}$. The identification of the ethylene peak was separately confirmed on other samples by repeating the injection after exposing the vessel atmosphere to potassium permanganate solution (0.1M), an ethylene absorber. Ethylene concentrations were calculated from peak area.

#### 4.2.4.2. Oxygen

Oxygen concentrations in the culture vessels were obtained by injecting samples into a specially made 'T' shaped glass chamber (Fig. 4.01a) fitted at one end with an oxygen micro-electrode (Clark type - tip diameter 10 µm)(Fig. 4.01b). The other end of the chamber was sealed with a rubber bung. The T-branch of the chamber was sealed with a 'Suba-seal' through which gas samples were normally injected.

The details of the design and construction of the 'Clark type' electrode have been described by Armstrong (1994) where a gold-plated low-melting point alloy was used as the cathode and a silver wire as the anode. To standardise the electrode it was polarised at a plateau potential (approx. -0.65V) to obtain the electrolysis current at air-saturation. The gas samples (1 ml) from the culture head-space were removed by a hypodermic syringe and then injected into the chamber. The change in the oxygen electrolysis were then recorded. Calibration is usually linear with a zero oxygen content correspondingly giving zero residual current.



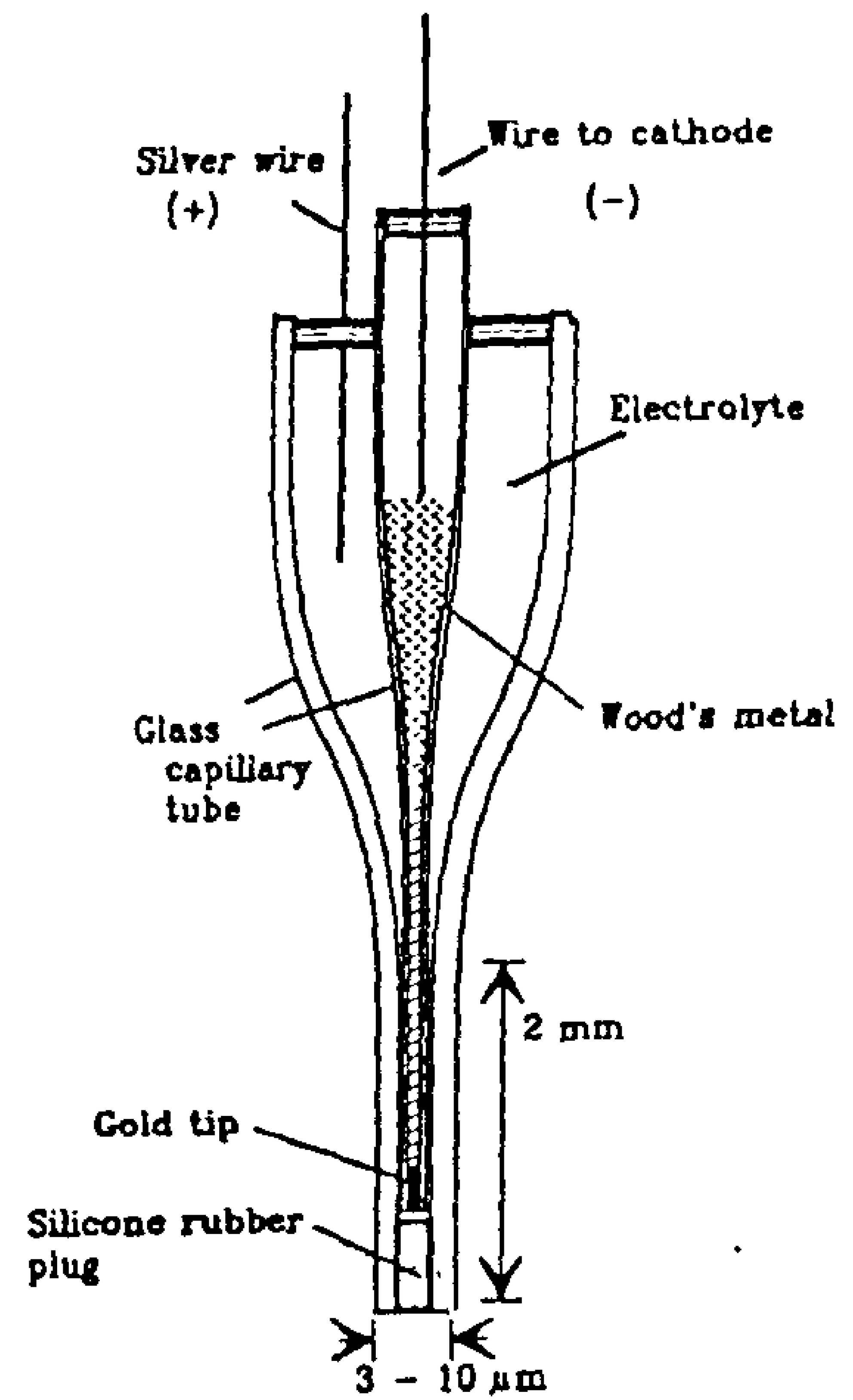




**Fig. 4.01. A) Specially made glass chamber fitted with an oxygen electrode to measure oxygen concentration of the culture vessel; B) schematic diagram of a 'Clark-type' electrode (Armstrong 1994)**

#### 4.2.4.3. Carbon dioxide

The carbon dioxide concentrations in the culture vessels were obtained by injecting (1 $cm^3$) gas samples into a closed circuit system (vol 40 $cm^3$) of an infra red gas analyzer (S. W. and W. S. Burrage, Hustingleigh, Ashford, Kent, UK) connected through a specially made chamber (Fig 4.02). Before injection the analyser had been calibrated using a 350 ppm $CO_2$ supply, and the subsequent injection samples were added after scavenging the IRGA $CO_2$ to zero.

### 4.2.5. Chlorophyll and carotenoid contents

Chlorophyll and carotenoid contents were determined when necessary as follows. Samples of chlorophyll from the fresh leaves of plantlets were prepared by homogenizing appropriate volumes of leaves in 80% acetone for 30s using an Ultra Turrax homogenizer, centrifuging at 300 rev. $min^{-1}$ for 10 min, and measuring light absorption between 400 and 700 nm in a spectrophotometer (Pye Unicam SP 1800). From the absorption curves, the proportion of chlorophyll a ($C_a$), chlorophyll b ($C_b$), and carotenoids ($C_{x+c}$), were evaluated according to formula of Lichtenthaler and Wellburn (1983).

### 4.2.6. Photosynthetic rate

The rates of $CO_2$ uptake/output by seedling/plantlets/callus were measured using the IRGA described above as a closed system into which the vessels were plugged using syringe needles and three-way taps. The system was originally charged with air ($CO_2$ concentration = 350 ppm) and this was circulated over the leaves by the pump system of the IRGA. The changes in $CO_2$ concentrations with time were recorded using a flat-bed recorder. The net atmospheric photosynthetic rate (APR), and that at the $CO_2$ levels created by the plants growing in that particular ventilating system (net working *in vitro* photosynthetic rate - IPR), were calculated in terms of mol $plantlet^{-1}$ $s^{-1}$ from the following equation:

$$\text{Photosynthetic rate} = \Delta C_a * V / (T * V_m * N),$$

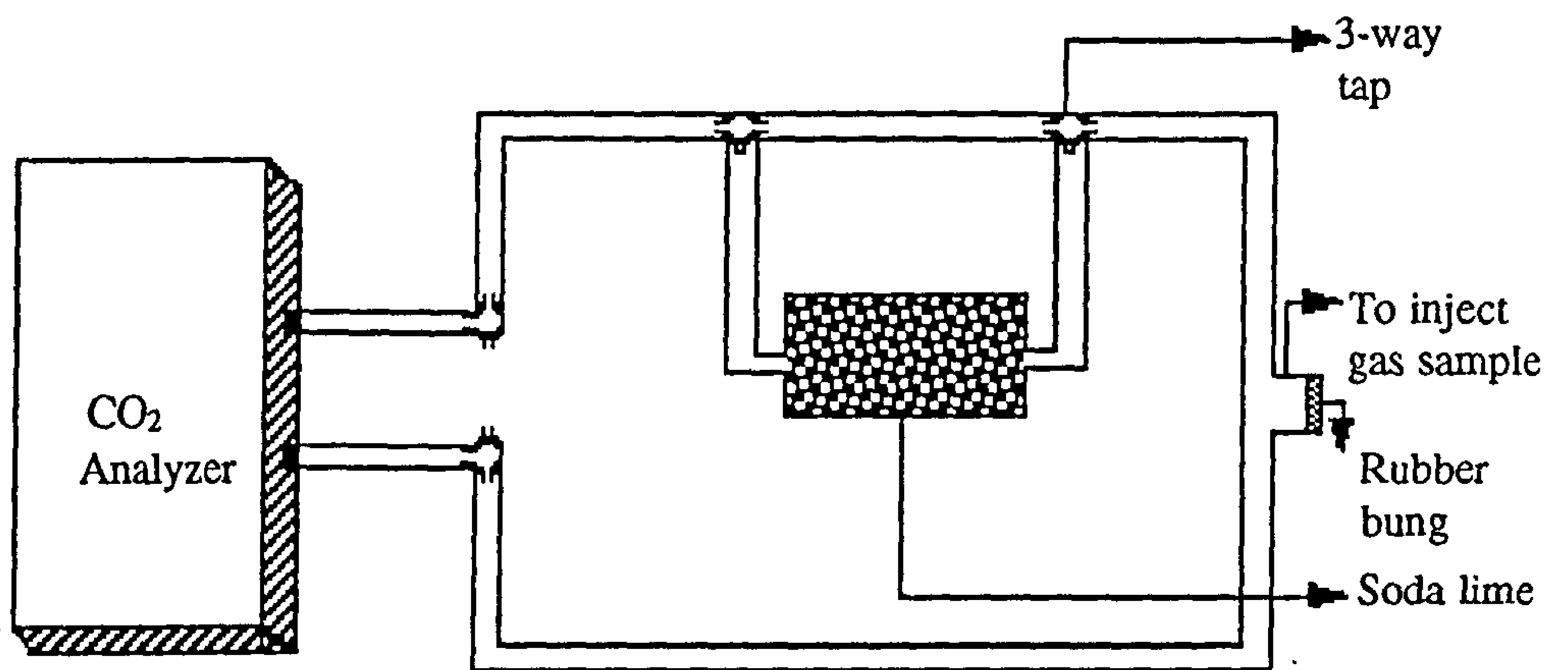



**Fig. 4.02 : Schematic diagram of a specially made chamber connected to an Infra red gas analyzer to measure $CO_2$ concentration of the culture vessels.**

where: $\Delta C_a$ is the change in $CO_2$ concentration (ml $ml^{-1}$) over time interval T (s) over which $CO_2$ concentration changes were recorded, V is the volume (ml) of the system + the volume of the culture vessel, $V_m$ is the molar volume of $CO_2$ at the growth room temperature, and N is the number of plantlets in the vessel. For APR the initial slope of the $CO_2$ *versus* time decay curve was used, while for IPR, a tangent to the slope was drawn at the 'working' $CO_2$ concentration. Rates were also expressed as mol $m^{-2}$ leaf surface $s^{-1}$ by substituting total leaf surface area ($m^2$) for N in the equation or as mol $m^{-3}$ $s^{-1}$ by substituting plantlet / calli volumes.

Leaf areas were measured on harvesting using a digital leaf area meter (Lambda Instruments Corporation). Plantlet and calli volumes were measured by sinking the plant material in the water in a measuring cylinder.

### 4.2.7. Experiments

#### 4.2.7.1. Effects of presence and absence of various ethylene inhibitors and precursors on growth and development in closed system

Silver nitrate ($AgNO_3$ at 5, 10 and 20 μM), cobalt chloride ($CoCl_2$ at 5, 10 and 20 μM), and silver thiosulphate (STS at 1, 5 and 10 μM) were used as ethylene inhibitors, and the ethylene precursor ACC (at 5, 10 and 20 μM) as an ethylene stimulator, were used at the different concentrations in the MS medium (Appendix 2). Surface sterilized seeds were inoculated on to the culture tubes containing 10 ml of the MS medium (4 seeds per tube) and placed in continuous light (PAR 70 μmol $m^{-2}$ $s^{-1}$) in the growth room to germinate and grow. There were 10 replicates per treatment.

Ethylene concentrations in the head-spaces of the culture vessels were measured (5 replicates per treatment) after 7 days. Growth measurements were performed after 18 days. These included leaf number, fresh weight and area, stem fresh weight, length and diameter and root number and maximum length.

**4.2.7.2. Growth and physiology of seedlings: the evaluation of the closed, diffusive and forced ventilation systems and the effects of $AgNO_3$ and ACC.**

Silver nitrate (at 10 μM) or ACC (at 2 μM) were added to the medium (MS with 3% sucrose) as required after filter sterilization (Millipore, 0.22 μm). Four seeds were inoculated per tube; there were 5 replicates per treatment and placed in the growth room in continuous light.

The following growth conditions were compared :

The vessels were: (a) sealed with silicone rubber bung (sealed control); (b) sealed with silicone rubber bung + $AgNO_3$ (10 μM) added in the medium; (c) sealed with silicone rubber bung + ACC (2 μM); (d) the vessels were capped with polypropylene membrane (diffusive control); (e) capped with polypropylene membrane + $AgNO_3$ (10 μM); (f) capped with polypropylene membrane + ACC (2 μM); (g) capped with a Slow Flow (SF)-ventilation apparatus (1.5 $cm^3$ $min^{-1}$) (SF-control); (h) capped with SF-ventilation apparatus + $AgNO_3$ (10 μM);. (i) capped with SF-ventilation apparatus + ACC (2 μM); (j) capped with a Fast Flow (FF)-ventilation apparatus (3.5 $cm^3$ $min^{-1}$) (FF-control); (k) capped with FF-ventilation apparatus + $AgNO_3$ (10 μM); (l) capped with FF-ventilation apparatus + ACC (2 μM).

Ethylene and $CO_2$ concentrations in the culture head-space were measured on the 7th and 12th days respectively; photosynthetic rates were measured on 12-day-old seedlings; growth and chlorophyll contents were measured on the 18th day. Also in the sealed condition (with and without $AgNO_3$ and ACC) ethylene concentrations were measured from days 1 - 12. In an additional experiment: sealed condition + ACC (2 μM), $CO_2$ concentrations were measured from days 1 - 20.

**4.2.7.3. Effects of different types of ventilation on growth and physiology of shoot cuttings**

Seeds were surface sterilized and inoculated in half strength MS medium and incubated under growth room conditions. Hypocotyl cuttings (5-7 mm in lengths) were obtained

from five days old seedlings; the cotyledons were dissected out and discarded. Cuttings were inoculated in 60 $cm^3$ culture vessels containing 10 ml of full strength MS medium supplemented with BAP (1.0 mg $l^{-1}$) and NAA (0.5 mg $l^{-1}$); this led to new shoot proliferation along with callus development. Each vessel was capped in one of the following ways: (a) a silicone rubber bung to seal the container, (b) a polypropylene disc to allow diffusive ventilation, or (c) a FF-ventilation apparatus for forced ventilation.

Only one explant was inoculated per vessel. Five replicates were prepared for each treatment. Cultures were kept in growth room conditions.

Concentrations of $CO_2$, $O_2$ and ethylene were measured throughout the experiment to the 30th day. Ethylene production rate for plant + callus, and callus alone were measured across 48 hours in the light on the 30th day. Photosynthetic rates and $CO_2$, production rates were measured on the 30th day. (For determining ethylene production rates, the culture vessels were uncapped to allow them to equilibrate with the atmosphere. For photosynthetic rates and $CO_2$ production rates the vessels were first charged with air). Growth of plants and callus and leaf chlorophyll and carotenoid contents were measured on the 31st day.

## 4.3. RESULTS AND DISCUSSION

### 4.3.1. Effects of ethylene inhibitors and precursors on seedling growth in the closed condition

#### 4.3.1.1. Ethylene inhibitors : $AgNO_3$, $CoCl_2$ and $Ag_2S_2O_3$

Compared to the additive-free controls, the addition of ethylene inhibitors in the media was found to have had significant stimulatory effects on the growth and development of cauliflower seedlings (Table 4.01; Plate 4.01). The best response was observed with 10 μM $AgNO_3$ in the medium, and the fresh weight of leaves was 2.6X, and the leaf area 2.8X those of the control. Here also the number of leaves was slightly higher than in the other treatments. The best root systems were also found in this treatment and the number of roots was 3.5X and maximum length of roots 2.2X those of the control. The fresh weights of the stems were also very much higher.

The other inhibitor of ethylene action, STS, also stimulated the growth and development of the seedlings. STS at 5.0 μM concentration produced the best performance for leaf fresh weight and area, but stem fresh weights and root numbers were greatest in the 10 μM treatment. Apart from leaf number most other growth parameters were substantially greater than those of the control. In the 5μM treatment, leaf areas and fresh weights were approx. twice those of the controls. The numbers of roots in the 10 μM treatment were approx. 3X higher than in the control.

The addition of cobalt in the form of cobalt chloride which inhibits ethylene formation also produced stimulatory effects on growth. The overall best performance was observed at the 20 μM concentration. The most significant development was observed in the leaf system where leaf fresh weight per seedling was 2.4X and leaf area 2.6X that of the control. The root systems were also better than the controls with root numbers at 20 μM concentration being approx. 3X those of the controls.

The ethylene concentrations in the culture vessels were measured during the experiment (Fig. 4.03). Significant amounts of ethylene were found in the head-space of the culture vessels when silver was added either in the form of $AgNO_3$ or STS. With

**Table 4.01.** **Effects of ethylene inhibitors and precursor on growth and development of cauliflower seedlings.**

| Treatments | | Leaf | | | Stem | | | Root | |
|---|---|---|---|---|---|---|---|---|---|
| | | F.W. (mg) | Number | Area ($cm^2$) | F.W (mg) | Length (mm) | Diameter (mm) | †Max. length (mm) | Number |
| **Control** | - | 112.1±13.2 | 5.3±0.3 | 3..5±0.1 | 15.5±2.7 | 12.9±0.7 | 0.90±0.02 | 29.0±1.1 | 6.1±0.4 |
| **ACC** | 5.0 μM | 66.9±9.2 | 4.8±0.2 | 2.4±0.1 | 36.4±1.2 | 11.4±0.8 | 1.8±0.2 | 17.3±5.0 | 5.0±1.1 |
| | 10 μM | 76.8±1.2 | 4.7±1.3 | 1.6±0.1 | 35.1±0.5 | 9.7±1.5 | 2.1±0.1 | 9.9±1.0 | 3.0±0.2 |
| | 20 μM | 34.3±1.4 | 2.7±0.8 | 0.7±0.1 | 23.1±0.5 | 7.4±1.3 | 2.0±0.1 | 7.4±0.4 | 3.2±0.3 |
| **$AgNO_3$** | 5.0 μM | 219.9±10 | 4.0±0.2 | 6.8±0.9 | 12.3±2.7 | 11.9±0.2 | 1.1±0.1 | 53.2±3.7 | 12.0±1.2 |
| | 10 μM | 289.8±14 | 5.7±0.5 | 9.9±0.9 | 23.1±0.9 | 13.7±1.9 | 1.2±0.1 | 62.9±1.8 | 21.4±0.3 |
| | 20 μM | 212.0±11 | 4.1±0.8 | 6.7±0.9 | 11.1±0.5 | 9.4±1.3 | 1.6±0.1 | 57.4±0.4 | 11.2±0.3 |
| **$Ag_2S_2O_3$** | 1.0 μM | 154.1±14 | 4.9±0.3 | 4.2±0.3 | 14.1±0.3 | 13.2±0.9 | 1.1±0.2 | 56.7±3.4 | 9.60±1.1 |
| | 5.0 μM | 229.8±10 | 4.0±0.2 | 7.6±0.7 | 18.3±2.0 | 13.5±0.5 | 1.1±0.1 | 46.2±3.1 | 14.0±1.8 |
| | 10 μM | 180.8±5.9 | 5.1±0.5 | 5.8±1.4 | 20.1±0.9 | 13.7±1.9 | 1.1±0.1 | 33.9±1.8 | 18.9±0.6 |
| **$CoCl_2$** | 5.0 μM | 124.1±11 | 4.8±0.8 | 3.1±0.8 | 14.1±0.9 | 12.2±0.9 | 1.0±0.1 | 39.7±2.4 | 6.60±1.1 |
| | 10 μM | 209.8±10 | 5.0±0.2 | 5.9±0.4 | 21.3±2.0 | 13.5±0.9 | 1.2±0.1 | 66.2±3.9 | 17.0±1.8 |
| | 20 μM | 270.8±5 | 5.6±0.5 | 9.2±1.0 | 30.1±0.5 | 12.7±1.0 | 1.6±0.1 | 49.9±1.9 | 17.9±0.4 |

*60 $cm^3$ culture vessels each containing four 18 days old seedlings; vessels were sealed with silicone rubber bungs; each value represents a mean ± SE of 20 seedlings; †mean maximum root length (3 roots from each seedling). Seedlings grown at *ca.* 25°C in continuous light ( PAR = 150 μmol $m^{-2}s^{-1}$).

## PLATE : 4.01

Showing the effects of ethylene inhibitors ($CoCl_2$, $AgNO_3$ and $Ag_2S_2O_3$) and precursor (ACC) on growth and development of cauliflower seedlings (18 days old) under sealed conditions.

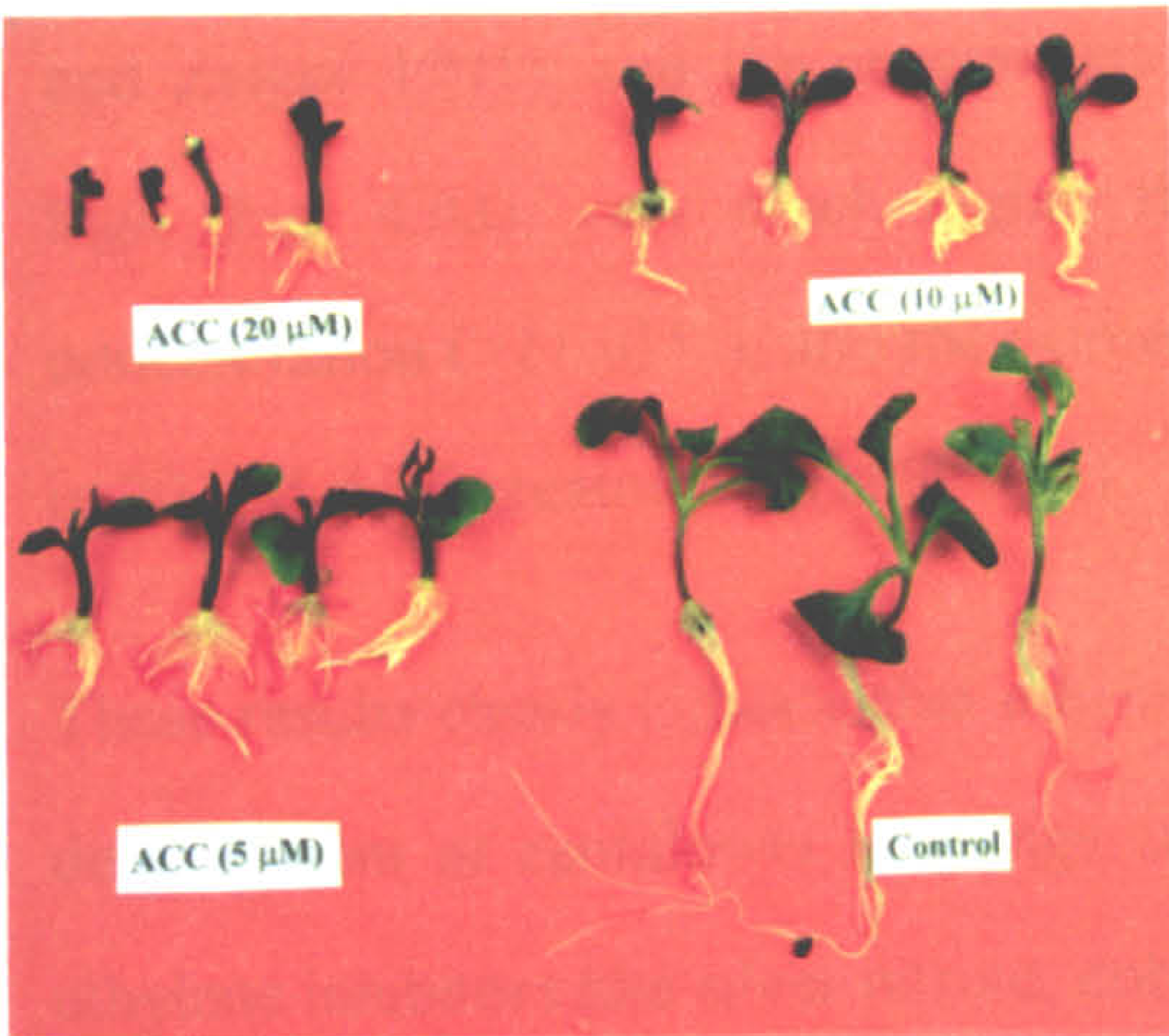
ACC treatment: note growth inhibition by ACC; best growth with ACC was at concentration of 5.0 µM.

$CoCl_2$ treatment: note stimulation of growth at 10 µM (best root) and at 20 µM (best shoot). Growth at 5.0 µM was almost the same as in control.

$AgNO_3$ treatment: note stimulation of growth at all concentrations used. Best growth was at 10 µM.

$Ag_2S_2O_3$ treatment: note stimulation of growth at all concentrations used but not as great as with $AgNO_3$. Best growth was at 5.0 µM. (Note rather thick laterals were apparently induced by 10 µM $Ag_2S_2O_3$.

Leaf epinasty was present in the controls and in ACC treatments and was largely prevented by $AgNO_3$ and 5.0 µM $Ag_2S_2O_3$.


ACC (20 μM)
ACC (10 μM)
ACC (5 μM)
Control

CoCl$_2$ (20 μM)
CoCl$_2$ (10 μM)
CoCl$_2$ (5 μM)
Control

AgNO$_3$ (5 μM)
Control

AgNO$_3$ (20 μM)
AgNO$_3$ (10 μM)

Control
Ag$_2$S$_2$O$_3$ (10 μM)

Ag$_2$S$_2$O$_3$ (5 μM)
Ag$_2$S$_2$O$_3$ (1 μM)

was only 7.4 mm which was 0.3X that of the controls. The leaf fresh weights, numbers and leaf areas in this treatment were only 0.3X, 0.5X and 0.2X respectively those of the controls. However stem fresh weights and diameters were substantially increased in the ACC treatments compared to the controls (Table 4.01, Plate 4.01).

All these effects on growth would seem to be typical responses to ethylene (Taiz and Zeiger 1991), and the concentrations measured during the experiment were very high (Fig 4.03), very much higher than in the additive-free controls. The highest concentration (>11 $\mu l\ l^{-1}$), and the poorest growth recorded after 15 days of culture, was in 20 μM ACC treatment (Table 4.01).

These results clearly indicate that ethylene can have inhibitory effects on the growth and development of cauliflower seedlings and that this can be overcome by the addition of ethylene inhibitors like silver nitrate, cobalt chloride or silver thiosulphate in the medium. The concentration of 10 μM $AgNO_3$ in the rooting medium appeared to best for counteracting the inhibitory effects of ethylene.

### 4.3.2. Growth and physiology of seedlings: the effects of closed, diffusive and forced ventilation systems with and without $AgNO_3$ or ACC in the culture medium

#### 4.3.2.1. Growth (no additives)

Taking account of all the parameters of growth and development within the various treatments, it is evident that seedlings grown in either diffusive or forced ventilation produce more vigorous shoot and root systems than those grown in sealed containers (Table 4.02, Plate 4.02).

The diffusive and SF-ventilations produced almost the same growth of leaf, shoot and root. The best growth by far occurred with the FF-ventilation with larger leaf areas (2.3X) and higher leaf fresh weights, (2.5X) those of the controls. Better root systems were also observed with the FF-ventilation with root length and number twice as great as for the controls; root numbers were also greater than in the diffusive system.

**TABLE 4.02.** **Effects of different types of ventilation, ACC and $AgNO_3$ on the growth and development of cauliflower seedlings.**

| Treatments | Methods of ventilation | Leaf | | | Stem | | | Root | |
|---|---|---|---|---|---|---|---|---|---|
| | | F.W. (mg) | Number | Area ($cm^2$) | F.W (mg) | Length (mm) | Diameter (mm) | †Length (mm) | Number |
| **Control** | Sealed | 84.1±1.4 | 5.0±0.8 | 2.6±0.7 | 11.1±0.3 | 9.2±0.9 | 0.76±0.1 | 36.8±3.4 | 5.6±1.3 |
| | Diffusive ventilation | 127.5±4.7 | 4.9±0.2 | 4.3±0.4 | 7.8±1.1 | 7.4±0.4 | 0.85±0.1 | 67.5±3.9 | 6.6±1.1 |
| | Forced ventilation (Slow) | 126.1±5.6 | 5.0±0.7 | 4.5±1.1 | 7.7±0.3 | 7.5±0.5 | 0.92±0.1 | 71.2±2.7 | 7.8±1.3 |
| | Forced ventilation (Fast) | 209.5±12 | 6.2±0.9 | 6.1±1.1 | 12.5±4.4 | 9.9±1.3 | 0.95±0.2 | 74.3±3.5 | 10.8±2.7 |
| **ACC** (2.0 μM) | Sealed | 78.5±2.2 | 6.7±0.8 | 2.0±0.1 | 19.2±0.4 | 11.1±0.5 | 1.4±0.1 | 23.3±1.5 | 3.0±0.2 |
| | Diffusive ventilation | 119.8±2.2 | 5.3±0.5 | 3.3±0.6 | 9.8±1.5 | 6.7±0.5 | 1.1±0.2 | 23.9±2.0 | 4.0±0.3 |
| | Forced ventilation (Slow) | 134.3±3.0 | 5.2±0.8 | 4.6±0.8 | 13.1±0.5 | 8.4±1.2 | 1.1±0.1 | 24.0±0.4 | 4.2±0.8 |
| | Forced ventilation (Fast) | 215.0±1.8 | 6.2±0.8 | 6.5±1.4 | 13.5±3.3 | 10.9±2.0 | 1.0±0.2 | 67.5±3.3 | 8.7±1.5 |
| **$AgNO_3$** (10 μM) | Sealed | 136.1±10.1 | 5.1±0.8 | 4.2±0.9 | 10.2±1.1 | 6.1±0.1 | 0.9±0.1 | 75.6±2.8 | 6.8±0.5 |
| | Diffusive ventilation | 138.8±4.2 | 5.3±0.6 | 4.4±0.6 | 9.5±0.6 | 6.6±0.7 | 0.9±0.1 | 77.7±9.9 | 8.8±0.8 |
| | Forced ventilation (Slow) | 156.0±7.2 | 5.3±0.7 | 5.6±1.1 | 11.2±1.1 | 8.6±0.7 | 0.8±0.1 | 78.9±6.1 | 9.0±1.0 |
| | Forced ventilation (Fast) | 219.7±8.5 | 6.3±0.6 | 7.0±0.9 | 12.5±1.1 | 10.5±0.8 | 0.9±0.1 | 82.5±7.7 | 11.0±1.3 |

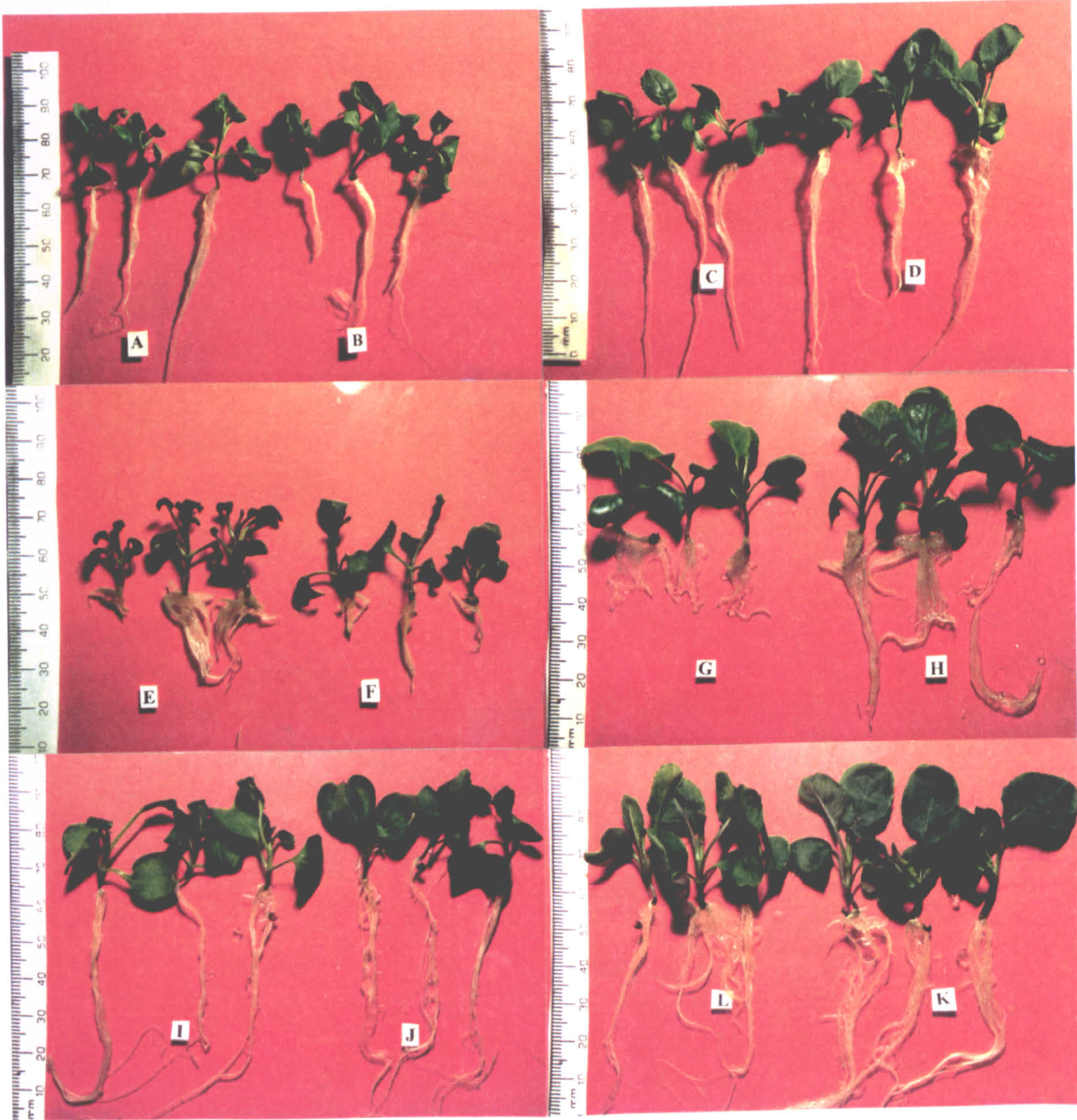
*12 days old seedlings (four seedlings per 60 $cm^3$ culture vessel); each measurement is for the best 2 (out of 4) seedlings from each of 10 tubes and represents a mean ± SE of 20 seedlings.
*Flow rates of slow and fast flow ventilations were 1.0 $cm^3$ $min^{-1}$ and 3.5 $cm^3$ $min^{-1}$ respectively; for sealed and diffusive ventilation vessels were capped with silicone rubber bungs and polypropylene discs respectively; Seedlings grown at *ca.* 25°C in continuous light; PAR = 150 μmol $m^{-2}s^{-1}$;
†mean maximum root length (3 roots from each seedling).

## PLATE : 4.02

Showing the effects of different types of ventilation on growth and development of cauliflower seedlings (18 days old).

(A) sealed (no additives);
(B) capped with polypropylene disc (no additives);
(C) capped with slow flow ventilation apparatus (no additives);
(D) capped with fast flow ventilation apparatus (no additives);
(E) sealed + ACC (2 μM);
(F) capped with polypropylene disc + ACC (2 μM);
(G) capped with slow flow ventilation apparatus + ACC (2 μM);
(H) capped with fast flow ventilation apparatus + ACC (2 μM);
(I) sealed + $AgNO_3$ (10 μM);
(J) capped with polypropylene disc + $AgNO_3$ (10 μM);.
(L) capped with slow flow ventilation apparatus + $AgNO_3$ (10 μM);
(K) capped with fast flow ventilation apparatus + $AgNO_3$ (10 μM);

Note in the control (A - D) and ACC treatments (E - H) growth increased with the efficiency of ventilation. Leaf epinasty in sealed and diffusive ventilation occurred in both these treatments.
$AgNO_3$ stimulated growth in all the treatments but the biggest effects was seen in the sealed and the diffusive treatments, where there was also an absence of leaf epinasty. Growth was similarly good with fast flow ventilation in both ACC and $AgNO_3$ treatments.



A
B
C
D
E
F
G
H
I
J
L
K

#### 4.3.2.2. Growth (with and without ACC)

Compared to the additive-free treatment, the ACC treatment with sealed ventilation produced some slight growth inhibition in the leaves but marked inhibition in root growth. Similar effects can be seen when comparing the additive-free and ACC diffusive treatments. In terms of root growth the same can be said of the SF-treatments, but ACC may have stimulated stem growth in the SF-treatment. However, the deleterious effects of ACC on growth and development of cauliflower seedlings appear to have been largely overcome by FF- ventilation (Table 4.02) with leaf fresh weights and areas and stem fresh weights equal to those of the additive-free treatment, and root numbers and lengths almost the same. As regards root development only the FF-ventilation was able to overcome the substantial root-growth inhibition found with ACC addition. However, it should be noted that the ACC concentration used was <0.5 times that of the lowest of the concentrations used in the previous experiment. The reason for this was because the ethylene concentrations found in that experiment were so very much higher than in the additive-free controls. It was hoped that 2 μM ACC would produce less ethylene but still sufficient to cause inhibition.

#### 4.3.2.3. Growth (with and without $AgNO_3$)

In all except the FF-treatments the presence of $AgNO_3$ in the medium, led to better seedling growth than in the ACC or additive-free controls; this was especially evident when comparing plants from the sealed vessels. Seedlings grown under SF-ventilation showed slightly better growth than under diffusive ventilation but statistically the differences are barely significant (Table 4.02, Plate 4.02). With FF-ventilation, however, although growth was not significantly better than in the additive-free control it is substantially better than in the sealed, diffusive or SF-ventilation treatments.

The increased growth even in the sealed system indicates that the silver ion behaved as expected as an ethylene inhibitor. Consequently, since no net photosynthesis is possible in the sealed systems (see later) and growth is entirely dependent upon the

sucrose in the culture medium, one may deduce that the difference in growth between the sealed and diffusive $AgNO_3$ treatments on the one hand (where leaf fresh weights were approx. 137 mg $plant^{-1}$) and the additive-free sealed controls on the other (leaf fresh weights approx. 84 mg $plant^{-1}$) can be attributed to ethylene inhibition of growth. Further it seems almost certain that the further improved growth under FF-ventilation will be a function of the improved $CO_2$ supply enabling the plants to benefit also from photosynthetic assimilate production.

It should also be mentioned that the root systems in the silver treatments in all conditions (sealed, diffusive and forced ventilation) were best among the three treatments (silver, ACC and control) (Table 4.02). The results also indicate that the addition of ACC or silver has little effect on growth when the vessels were ventilated forcibly The leaf, shoot and the root systems of these two treatments were almost the same as those of the control when forced ventilation was applied in the culture vessels. Therefore, it can be concluded that the growth and development of cauliflower plants can be very significantly improved by applying forced ventilation in the culture vessels. These results are consistent with the findings of Kozai, Kubota, and Nakayama (1989) where better growth and higher photosynthetic rates of *in vitro* grown strawberry plants were found by applying forced ventilation. Yue, Gosselin and Desjardins (1993) also confirmed that forced ventilation (at a rate of 100 ml/min through each culture vessel; vol.= 340 $cm^3$) can improve the growth of geranium plantlets. In the present study rates of 1 - 5 ml/min were used, but in much smaller vessels (vol. = 60 $cm^3$).

#### 4.3.2.4. Photosynthesis (with and without ACC or $AgNO_3$)

Photosynthetic rates were measured throughout the range of treatments in the present experiment and are presented in Table 4.03. The rates were those achieved (i) at atmospheric $CO_2$ levels (i.e. net atmospheric photosynthetic rate - APR) where the vessels were temporarily charged with atmospheric air, and (ii) at the $CO_2$ levels created by the plants in that particular ventilating system (net working *in vitro* photosynthetic rate - IPR).

**Table 4.03.** Effects of different types of ventilation, ACC and $AgNO_3$ on photosynthetic rates of cauliflower seedlings (12 days old).

| Treatments | Ventilation | *Net photosynthetic | atmospheric rate (APR) | †Net working photosynthetic | *in vitro* rate -IPR |
|---|---|---|---|---|---|
| | | *pmol plant$^{-1}$ s$^{-1}$* | µmol m$^{-2}$ s$^{-1}$ | *pmol plant$^{-1}$ s$^{-1}$* | µmol m$^{-2}$ s$^{-1}$ |
| **Control** | Sealed | 500 ± 10 | 2.10± 0.04 | ≤ 0 | ≤ 0 |
| | Diffusive ventilation | 990 ± 20 | 2.81 ± 0.02 | 310± 10 | 0.86 ± 0.01 |
| | Forced ventilation (slow) | 1200 ± 18 | 3.11± 0.03 | 550 ± 12 | 1.40 ± 0.03 |
| | Forced ventilation (fast) | 1410 ± 31 | 3.92 ± 0.08 | 850 ± 12 | 1.90 ± 0.04 |
| ACC | Sealed | - (349 ± 12) | - (1.47 ± 0.04) | ≤ 0 | ≤ 0 |
| (2.0 µM) | Diffusive ventilation | 980 ± 9 | 3.20 ± 0.04 | 161 ± 30 | 0.56 ± 0.01 |
| | Forced ventilation (slow) | 1090± 16 | 3.71 ± 0.09 | 480 ± 17 | 1.30 ± 0.01 |
| | Forced ventilation (fast) | 1130 ± 8 | 3.90 ± 0.09 | 760 ± 70 | 2.00 ± 0.02 |
| $AgNO_3$ | Sealed | 730 ± 50 | 2.90 ± 0.01 | ≤ 0 | ≤ 0 |
| (10 µM) | Diffusive ventilation | 750 ± 27 | 2.80 ± 0.04 | 160 ± 30 | 0.61 ± 0.02 |
| | Forced ventilation (slow) | 970 ± 9.9 | 3.80 ± 0.07 | 370 ± 40 | 1.20 ± 0.03 |
| | Forced ventilation (fast) | 1170 ± 15 | 3.90 ± 0.09 | 860 ± 50 | 2.10 ± 0.05 |

*Photosynthetic rates measured at 350 µl l$^{-1}$ $CO_2$ and 72 µmol m$^{-2}$ s$^{-1}$ light flux. †Photosynthetic rates measured at known $CO_2$ concentrations of the culture vessels during the experimental period.

❖Each value represents a mean ± SE of 5 replicates.

❖Flow rates of slow and fast flow ventilations were 1.0 cm$^3$ min$^{-1}$ and 3.5 cm$^3$ min$^{-1}$ respectively; for sealed and diffusive ventilation vessels were capped with silicone rubber bungs and polypropylene discs respectively; volume of culture vessel = 60 cm$^3$. Seedlings grown at *ca.* 25°C in continuous light; PAR = 150 µmol m$^{-2}$s$^{-1}$.

Each was measured in terms of $CO_2$ exchange as (a) mol $plant^{-1}s^{-1}$ and (b) mol $m^{-2}$ leaf surface $s^{-1}$ and calculated from tangents to the curves shown in Fig 4.04 (see also Table 4.05).

Considering first the APR data it is not surprising to find that, since plant size tended to increase with increasing efficiency of the ventilating system, the APR per plant shows a similar trend; however, variability blurred any possible differences between diffusive flow and SF-ventilation and between FF- and SF-ventilation. On a leaf area basis photosynthetic rates were very similar across most treatments, but the rate in the additive-free controls were significantly lower while the data for ACC sealed treatment were extraordinary. It is particularly interesting to note that the plants in the additive-free sealed systems were photosynthetically fully-functional, but that in the ACC sealed system not only was there no net photosynthesis but, when these vessels were charged with air and re-sealed into the closed circuit of the IRGA, there was a net output of $CO_2$. Perhaps this indicated an onset of senescence brought on by the prolonged exposure to high levels of ethylene (Table 4.04); in Fig. 4.04 it can be seen that the chlorophyll levels in these plants were very much lower than in the additive-free sealed treatment.

The IPR data are a closer reflection of the normal *in vitro* metabolism and here the very significant differences between each of the systems of ventilation become apparent, with the FF-system clearly providing by far the best conditions for assimilation. At this stage of growth (15 days), because of the improved $CO_2$ supply (Table 4.05), the rates of photosynthesis in the SF-systems (Fig. 4.04) were approximately double those with the diffusion-dependent ventilation, while FF-rates were substantially higher (= 1.4X) than rates under SF-ventilation; however, rates did not differ much between systems in equivalent additive and additive-free treatments except again in the case of the ACC sealed system. Net photosynthesis was zero in the sealed additive-free control and its $AgNO_3$ counterpart emphasizing the dependency of growth upon the sucrose in the culture medium. The net $CO_2$ output of the ACC sealed treatment has already been commented on. Again based both

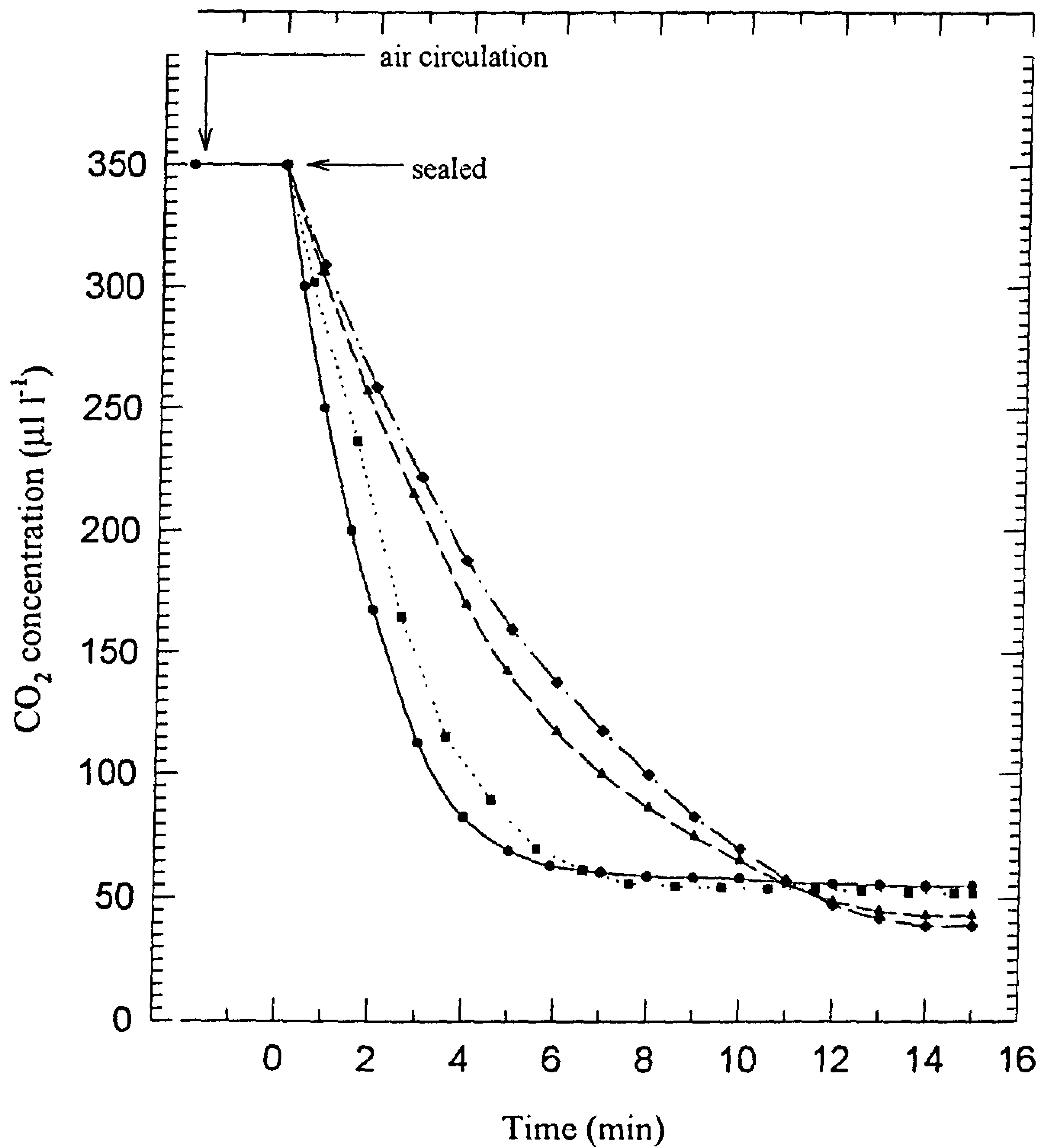



**Fig. 4.04.** Showing the depletion of carbon dioxide concentrations in the culture head-space in sealed condition (at 72 μmol m$^{-2}$s$^{-1}$ PAR) by the end of the experiment. 12 days old seedlings were previously grown under different types of ventilation (sealed - with silicone rubber bung - ◆, diffusive - capped with polypropylene disc - ▲, slow forced ventilation - flow rate = 1.0 cm$^3$ min$^{-1}$ - ■; and fast forced ventilation - flow rate = 3.5 cm$^3$ min$^{-1}$- ●). 60 cm$^3$ culture vessels each contained four seedlings, grown at *ca.* 25°C in continuous light; PAR = 150 μmol m$^{-2}$s$^{-1}$. Each symbol represents a mean ± SE of 5 replicates.

upon the photosynthetic rates per plant it is clear that the FF-ventilation system is superior to the other systems.

These results are consistent with the findings of some other authors who illustrated that enhancement of $CO_2$ in the culture vessels increased photosynthetic rate. Solárová *et al.* (1989) mentioned that successful cultivation needs an effective $CO_2$ supply inside the vessels, and in another investigation on the effects of forced ventilation on the growth of strawberry *in vitro* carried out by Kozai, Kubota, and Nakayama (1989) using PPF (photosynthetic photon flux) of 220 μmol $m^{-2}$ $s^{-1}$ and $CO_2$ concentrations of 350 - 2000 μl $l^{-1}$, higher photosynthetic rates and better growth were observed. They also suggested that forced ventilation could increase diffusion and assimilation of $CO_2$ by leaves. Yue, Gosselin and Desjardins (1993) observed that the growth of geranium was promoted by forced ventilation in which plantlets can carry out normal photosynthesis.

With regard to the effects of ethylene on photosynthesis, it seems possible that there may have been some direct effect in the additive-free controls through effects on chlorophyll contents (see Fig. 4.05). In the ACC sealed treatment the net $CO_2$ output in the light seems likely to have arisen from the long term influence of high ethylene levels. In forced ventilation ethylene cannot accumulate and $CO_2$ was enriched in the culture vessels' atmosphere, stimulating the higher photosynthetic rates which are reflected in the growth. It seems very likely that if the experiment had been prolonged the differences between the ventilating systems might have become even more accentuated. In summary, considering all the parameters of growth and development of these *in vitro*-grown cauliflower seedlings, it seems clear that the growth is best under forced ventilation (flow rate 3.5 $cm^3$ $min^{-1}$ during the experiment) which facilitates increased $CO_2$ supply and the removal of toxic gases like ethylene from the culture vessels. In Figure 4.04, it is likely that $CO_2$ levels with the seedlings from the forced ventilation treatments remained rather higher than with those from the sealed or diffusive treatments towards the end of the measurement-period. This was probably because with forced ventilation the plants

were larger and there was mutual shading of the leaves. This did not occur with the smaller plants from the latter two treatments.

#### 4.3.2.5. Chlorophyll content (with and without ACC or $AgNO_3$)

The lowest chlorophyll content based on the fresh weight of the leaves was observed with sealed ventilation + ACC (240 $\mu g\ g^{-1}$: Fig. 4.05); the highest values were in the FF-systems and were very much higher than this (4.3X), and were significantly greater than in the diffusive and SF-systems. In all cases SF-ventilation marginally increased chlorophyll contents above those with diffusive ventilation.

The addition of silver substantially increased the chlorophyll contents under sealed ventilation but the effect was only slight within diffusive or forced ventilation systems. The rise in chlorophyll content which accompanied the silver addition in the sealed vessels strongly suggests the countering of ethylene action. Similarly the very low chlorophyll content (240 $\mu g\ g^{-1}$) in the sealed vessels + ACC (ethylene 1.7 $\mu l\ l^{-1}$), and the low value (423 $\mu g\ g^{-1}$) in the additive-free sealed vessels (ethylene 0.3 $\mu l\ l^{-1}$), suggests that ethylene may have depressed the chlorophyll levels. Since ethylene was undetected in the SF and FF systems it seems possible that the much higher chlorophyll contents in the FF-systems might have been linked in some way to the more equable levels of $CO_2$ content under FF.

These findings were in agreement with the findings of Cournac *et al.* (1991) in *Solanum tuberosum* where in closed vessels lower chlorophyll contents were recorded. He also showed that the addition of silver in the form of $Ag_2S_2O_3$ increased chlorophyll contents slightly However, in aerated vessels chlorophyll contents also increased significantly and this is similar to the present findings.

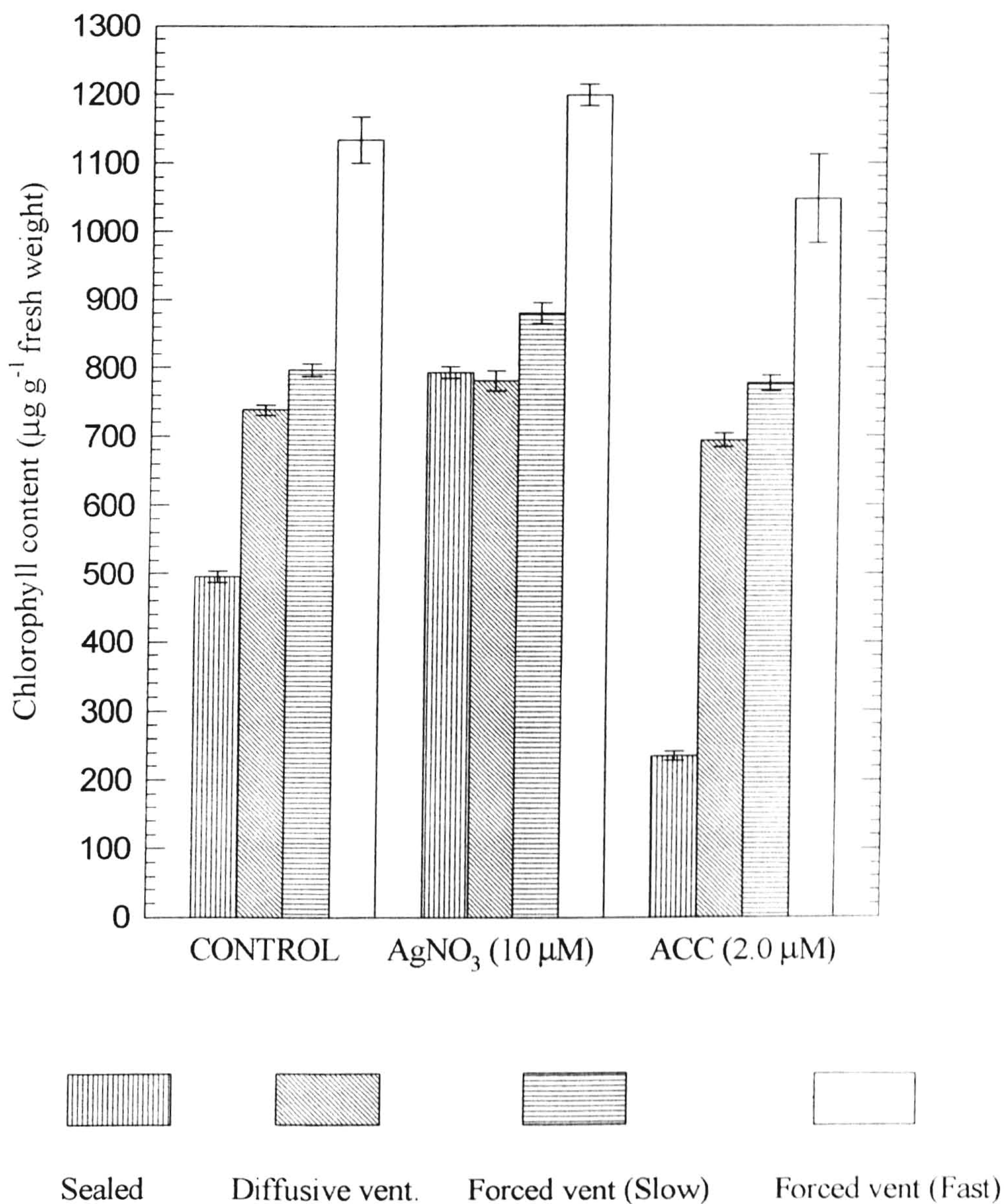



**Fig. 4.05.** Effects of different types of ventilation and the presence and absence of ethylene inhibitor ($AgNO_3$) and precursor (ACC) in the medium on chlorophyll contents of 18 days old cauliflower seedlings. For sealed and diffusive conditions culture vessels were capped with silicone rubber bungs and polypropylene discs respectively; the flow rates of slow and fast flow ventilations were 1.0 and 3.5 $cm^3$ $min^{-1}$. 60 $cm^3$ culture vessels each contained four seedlings, grown at *ca.* 25°C in continuous light; PAR = 150 μmol $m^{-2}$ $s^{-1}$. Each value represents a mean ± SE of 5 replicates.

### 4.3.3. Effects of different ventilation systems on head-space gas composition in the culture vessels

#### 4.3.3.1. Ethylene (with and without ACC or $AgNO_3$)

The results for the growth of seedlings (Table 4.02) were consistent with the findings of ethylene concentrations in the head-space of the culture vessels in the different treatments (Table 4.04). In sealed vessels, the addition of ACC to the nutrient medium resulted in very high concentrations of ethylene (1.70 µl $l^{-1}$) after 7 days of culture in comparison to the additive-free controls (0.32 µl $l^{-1}$) or the silver treatment (0.94 µl $l^{-1}$) (Table 4.04, Fig. 4.06). Since silver (the ethylene inhibitor) enhanced the growth it seems reasonable to conclude that the 0.32 µl $l^{-1}$ ethylene was sufficient to inhibit the plant growth. The value of 0.32 µl $l^{-1}$ was therefore probably due in large measure to the tiny size of the plants, and the 0.94 µl $l^{-1}$ in the presence of silver, a function of the larger leaf and root systems and the insensitivity to ethylene because of the presence of the silver. However, the presence of silver may stimulate some ethylene production. Roustan, Latche and Fallot (1991) have reported that the addition of silver ions to the medium led to slight increases in ethylene production by the embryonic cells. Similarly Khalid *et al.* (1991) found that high concentrations (25 µM) of silver nitrate in *Helianthus annus* cotyledon cultures slightly stimulated ethylene production.

The culture vessels with diffusive ventilation contained only low concentrations of ethylene: 0.09 µl $l^{-1}$ in the additive-free controls, 0.35 µl $l^{-1}$ with ACC and 0.18 µl $l^{-1}$ in the silver treatment. All these data showed that the concentrations of ethylene in containers with diffusive ventilation were significantly lower than those of the sealed ones. This is most likely to be because of the diffusive escape of ethylene through the polypropylene membrane of the culture vessels; probably, as a consequence, better growth was observed in this capping system compared to the sealed one.

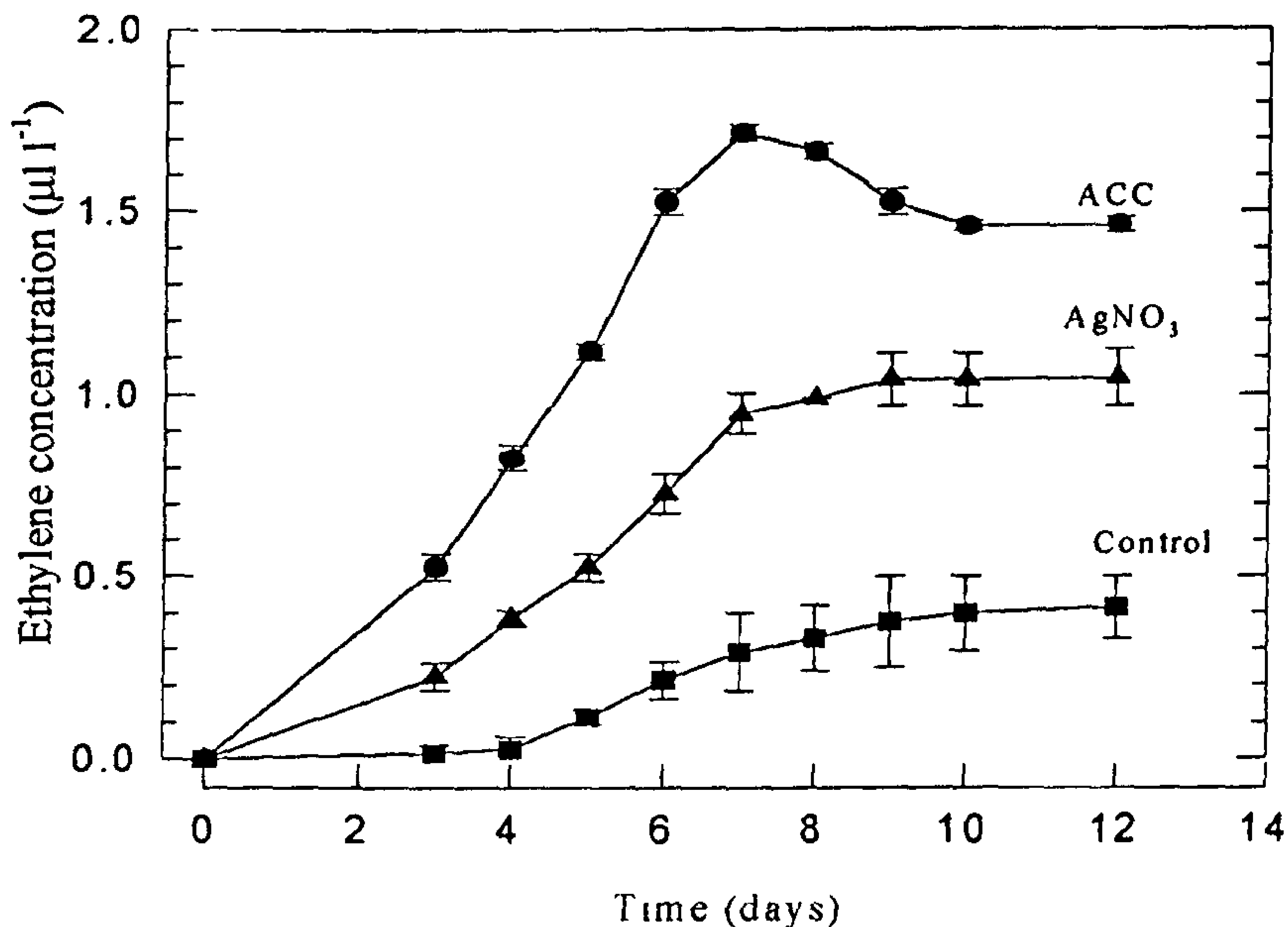



**Fig. 4.06.** **Showing the ethylene concentrations in culture head-spaces under sealed condition (silicone rubber bung) and in presence of ACC (2.0 µM) *or* $AgNO_3$ (10 µM) in the medium. 60 $cm^3$ culture vessels each contained four seedlings, grown at *ca.* 25°C in continuous light; PAR = 150 µmol $m^{-2}s^{-1}$. Each symbol represents a mean ± SE of 5 - 7 replicates.**

When the slow flow or fast flow ventilation apparatus was applied no ethylene was noticed in the head-space of the vessels. This indicates that the flushing out of the accumulated ethylene from the culture vessels may be partially responsible for the better growth compared with the sealed or diffusive ones. However, the ability of these ventilation systems to improve $CO_2$ concentration in the culture vessels might have been largely responsible for the better growth.

As shown in table 4.04 ethylene concentrations were relatively lower in the dark period compared with the light. This may have been due to high $CO_2$ accumulation in the head-space of the culture vessels during the dark period. Similar findings were reported by Adkins (1992) in rice callus where in the dark, the rate of ethylene production was much lower than in the light.

**Table 4.04.** Effects of different types of ventilation, ACC and $AgNO_3$ on ethylene concentration ($\mu l\ l^{-1}$) in the culture head-space of *in vitro* grown cauliflower seedlings.

| Treatments | | Sealed with silicone rubber bung | Diffusive ventilation (capped with polypropylene disc) | Forced ventilation (Slow flow) | Forced ventilation (Fast flow) |
|---|---|---|---|---|---|
| Control | Light | 0.32 ± 0.02 | 0.09 ± 0.01 | 0.0 | 0.0 |
| | Dark | 0.24 ± 0.06 | 0.06 ± 0.01 | 0.0 | 0.0 |
| ACC (2.0 μM) | Light | 1.70 ± 0.10 | 0.35 ± 0.02 | 0.0 | 0.0 |
| | Dark | 1.07 ± 0.15 | 0.19 ± 0.01 | 0.0 | 0.0 |
| $AgNO_3$ (10 μM) | Light | 0.94 ± 0.02 | 0.18 ± 0.03 | 0.0 | 0.0 |
| | Dark | 0.66 ± 0.03 | 0.08 ± 0.01 | 0.0 | 0.0 |

*Seedlings grown at *ca.* 25°C with continuous light period ( PAR = 150 $\mu mol\ m^{-2}s^{-1}$). Ethylene concentrations at dark were measured only on the 7th day of the experiment after 10 h darkness. Each value represents a mean ± SE of 7 - 10 replicates.

### 4.3.3.2. Carbon dioxide (with and without ACC or $AgNO_3$)

Carbon dioxide concentrations inside the vessels must depend upon the balance between respiration and photosynthesis and the efficiency of the vessel's ventilating system. In the present investigation when the vessels remained sealed, very high $CO_2$ concentrations in dark and very low concentrations in light were recorded (Table 4.05). With diffusive and/or forced ventilation there was still a pattern of higher $CO_2$ concentrations in the dark than in the light but the differences were much less. Generally in the dark $CO_2$ concentrations inside culture vessels increase due to respiration (Fujiwara, Kozai and Watanabe 1988, Jackson *et al.* 1991, Solárová *et al.* 1989) and during the light period decrease depending on the photosynthetic activity of the plants (Buddendorf, Josten and Woltering 1994). These findings accord with the present investigation where in the sealed condition respiratory $CO_2$ is accumulated significantly during darkness. However, with diffusive ventilation much $CO_2$ must escape by means of diffusion and with forced ventilation the $CO_2$ concentration in darkness is dominated by the flushing through of

humidified atmospheric air: thus $CO_2$ levels remained significantly lower than those in the sealed systems.

**Table 4.05. Effects of different types of ventilation, ACC and $AgNO_3$ on carbon dioxide concentrations ($\mu l\ l^{-1}$) in the culture head-space of *in vitro* grown cauliflower seedlings after 12 day.**

| Treatments | | Sealed with silicone rubber bung | Diffusive ventilation (capped with polypropylene disc) | Forced ventilation (Slow flow; rate = 1.0 $cm^3\ min^{-1}$) | Forced ventilation (Fast flow; rate = 3.5 $cm^3\ min^{-1}$) |
|---|---|---|---|---|---|
| Control | Light | 36.8±5 | 100.3±9 | 120.3±15 | 150±15 |
| | Dark | 27585±450 | 3979±78 | 1181±94 | 565±38 |
| ACC | Light | †38.9±9.0 | 90.7±15 | 100.7±23 | 149±23 |
| (2.0 μM) | Dark | 28590±503 | 4390±93 | 1270±82 | 569±43 |
| $AgNO_3$ | Light | 61.8±3.0 | 86.5±9.0 | 100.5±09 | 150.5±21 |
| (10 μM) | Dark | 27980± 493 | 4013±88 | 1225±88 | 559±31 |

*Seedlings grown at *ca.* 25°C in continuous light; PAR = 150 $\mu mol\ m^{-2}s^{-1}$. $CO_2$ concentrations in the dark were measured only on the 12th day of the experiment after 10 h darkness.
*Each value represents a mean ± SE of 7 - 10 replicates.
† $CO_2$ concentration on the 12th day which started increasing from 15th day and reached 7200 $\mu l\ l^{-1}$ on 20th day (see Figure 4.07).

In the light period due to photosynthetic activity, the $CO_2$ concentration normally decreases; this has been reported earlier by many authors e.g. Desjardins *et al.* 1988, Kozai *et al* 1987, Kozai and Iwanami 1988 and Solárová *et al.* 1989. In sealed vessels the effects may bring the $CO_2$ concentration of the culture vessels to a very low level (*ca.* 40 $\mu l\ l^{-1}$ in this experiment) which represents the $CO_2$ compensation point, and this is generally considered to be the limiting factor for plant growth in these circumstances (Buddendorf - Josten and Woltering 1994). Buddendorf - Josten and Woltering (1994) argued that the retarded plant growth in tightly sealed vessels might be due to $CO_2$ deficiency, and this finding is consistent with the results presented in Table 4.02 which showed the deleterious effects of sealed vessels. On the other hand evidence has been

presented here which shows that ethylene might have been the *immediate* cause of the reduced growth in the sealed vessels (section 4.3.3.1).

When the vessels were capped with polypropylene membrane as a diffusive ventilator, $CO_2$ concentrations increased significantly and possibly partly as a result better growth were observed (described earlier in Section 4.3.2.). These results are similar to the findings of Blazková *et al.* (1989) where they suggested that the diffusion of $CO_2$ into the vessel increased plant growth. Similarly, in forced ventilation the $CO_2$ concentration increased up to 150 $\mu l\ l^{-1}$ (Table 4.05) and as a result much more vigorous shoot and root systems were observed.

Where there had been an addition of $AgNO_3$ or ACC (except for the sealed system) to the medium, similar results were obtained. In the case of ACC addition in the sealed system the $CO_2$ levels in the light varied considerably from days 1 to 20, depending on the stages of development of the seedlings (Fig. 4.07). From days 1 to 4, during germination, the $CO_2$ levels rose from 350-5000 $\mu l\ l^{-1}$ as a result of respiration. From days 4 to 7 when the cotyledons unfolded and were photosynthesising, the $CO_2$ levels decreased to below the compensation point *ca.* 40 $\mu l\ l^{-1}$; these values were similar to those in the $AgNO_3$ and additive-free controls (data not shown). Around day 13 the levels again increased to reach 7200 $\mu l\ l^{-1}$ on the 20th day. This latter increase was thought to have been due to the accumulation of ethylene in the culture head-space, which impeded photosynthesis, so that respiratory $CO_2$ accumulated; this also probably indicated the first stages of ethylene-induced premature senescence.

### 4.3.4. Shoot culture : the effects of closed, diffusive and FF-ventilation systems

#### 4.3.4.1. Growth and development

Inoculating shoot tips on to culture medium led to new shoot proliferation and callus development. Shoot growth in FF-ventilation was considerably better than in the sealed vessels, with those from the diffusive ventilation treatment being intermediate between the two (Table 4.06). For example the fresh weight of the stems from the forced

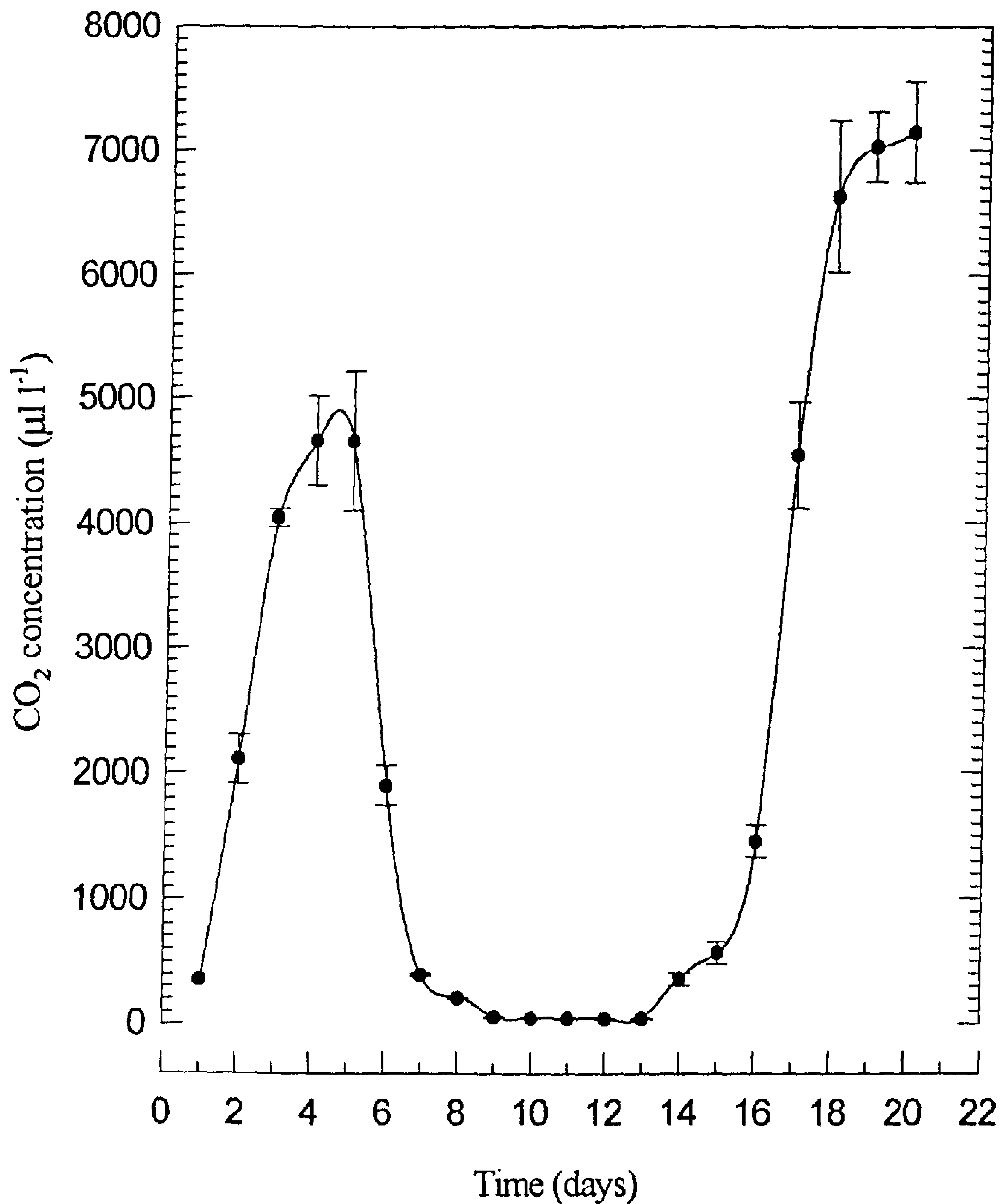



**Fig. 4.07.** Showing the changes in carbon dioxide concentrations in culture head-spaces under sealed condition (silicone rubber bung) and in the presence of ACC (2.0 µM) in the medium. 60 $cm^3$ culture vessels each contained four seedlings, grown at *ca.* 25°C in continuous light; PAR = 150 µmol $m^{-2}s^{-1}$. Each symbol represents a mean ± SE of 5 replicates.

Note the rise in concentration from days 1 - 4 during germination. Between days 4 - 7 the cotyledons unfolded and the $CO_2$ levels decreased to below the compensation point due to photosynthesis. After day 13 the levels increased to reach a very high concentration by day 20. This latter increase is thought to have been due to ethylene accumulation which impeded photosynthesis and induced premature senescence.

ventilation treatment was 5X that of the sealed controls and 3.4X that of diffusive ventilation. The most noticeable differences, however, were in the leaf systems, where the fresh weight of leaves in forced ventilation was almost 19X that of sealed control and 4.7X that in diffusive ventilation. There were similar differences between the treatments in respect of leaf numbers and areas.

**Table 4.06. Effects of different types of ventilation on growth and development of shoot cultures of cauliflower.**

| Treatment | Sealed (with silicone rubber bung) | Diffusive ventilation (Capped with polypropylene) | Forced ventilation (Flow rate 3.5 $cm^3 min^{-1}$) |
|---|---|---|---|
| **Morphology** | | | |
| **†Leaves** | | | |
| Numbers | 5.1±0.7 | 7.7±1.2 | 15.2±0.9 |
| F.W. (mg) | 37.4±3 | 149±7.6 | 695±9.0 |
| Area ($cm^2$) | 2.1±0.1 | 3.1±0.2 | 16.2±2.1 |
| **†Stem** | | | |
| Length (mm) | 6.4±0.1 | 7.8±0.9 | 12.9±1.2 |
| Diameter (mm) | 1.5±0.2 | 1.9±0.3 | 2.3±0.3 |
| F.W. (mg) | 29.0±3 | 45±3.5 | 153.0±3 |
| ***Callus** | | | |
| FW(mg) | 441±9.4 | 996±12 | 976±9.3 |
| Vol ($cm^3$) | 1.20±0.1 | 2.6±0.6 | 2.50±0.5 |
| ***Number of new shoots** | 3.1±0.3 | 3.6±0.6 | 3.5±0.5 |

†Each measurement is for best 2 shoots from each of 5 tubes and represents a mean ± SE of 10 replicates.
* each value represents a mean ± SE of 5 replicates.
60 $cm^3$ culture vessels each contained one cutting. Cultures were grown at *ca.* 25°C in continuous light; PAR = 150 $\mu mol\ m^{-2}s^{-1}$.

The texture of the calli in vessels with forced ventilation as well as with diffusive ventilation was friable and without any necrotic spots, whereas in the sealed vessels the calli bore a number of necrotic spots and became compact in texture. The colour of the calli grown in forced ventilation was greenish and/or creamy, whereas in the diffusive ventilation and sealed vessels calli became greyish. The volumes and fresh weights of

calli in forced and diffusive ventilations were significantly higher than those from sealed vessels (Table 4.06).

### 4.3.4.2. Photosynthesis

After 30 days of culture no net photosynthesis was shown by plantlets grown under diffusive ventilation or in the sealed condition were (Table 4.07). This may have been due to the vitrification of plants caused by high ethylene and low oxygen concentration in the atmosphere of the culture vessels in sealed and diffusive condition. The plants subjected to forced ventilation showed a net APR photosynthetic rate of 4.52 $\mu$mol $m^{-2}s^{-1}$ and an IPR of 0.81 $\mu$mol $m^{-2}$ $s^{-1}$. Similar findings in strawberry were reported by Kozai, Kubota and Nakayama (1989) where forced ventilation improved the photosynthetic rates of plantlets.

**Table 4.07. Effects of different types of ventilation on photosynthetic rates of 30 days old cauliflower cultures (shoots + callus) expressed on a leaf area basis.**

| Treatments | *Net atmospheric photosynthetic rate -APR ($\mu$mol $m^{-2}s^{-1}$) | †Net working *in vitro* photosynthetic rate -IPR ($\mu$mol $m^{-2}s^{-1}$) |
|---|---|---|
| **Forced ventilation (fast flow - 3.5 $cm^3$ $min^{-1}$)** | 4.52±0.9 | 0.81±0.05 |
| **Diffusive ventilation (capped with a polypropylene disc)** | ≤ 0.0 | ≤ 0.0 |
| **Sealed with silicone rubber bung** | ≤ 0.0 | ≤ 0.0 |

* Photosynthetic rates measured at 350 ppm $CO_2$ and 72 $\mu$mol $m^{-2}$ $s^{-1}$ light flux.
† Photosynthetic rates measured at known $CO_2$ concentrations of the culture vessels during the experimental period.
* Each value represents a mean ± SE of 5 replicates. Cultures were grown at *ca.* 25°C in continuous light; PAR = 150 $\mu$mol $m^{-2}s^{-1}$. 60 $cm^3$ culture vessels each contained one cutting.

#### 4.3.4.3. Chlorophyll and Carotenoid contents

The lowest chlorophyll content based on leaf fresh weight was observed in tightly sealed vessels (Table 4.08). Under forced ventilation it was very much greater: 3.3X and 2.9X higher than from the sealed and diffusive systems respectively.

The lowest ratio of chlorophyll a/b, (0.54), was found in plantlets grown in tightly sealed vessels; in those with diffusive and forced ventilation the ratios were significantly higher (1.2 and 1.4 respectively).

Carotenoid content was also noticeably higher in forced ventilated (120 mg/g fresh weight) vessels; this compares with 69.5 and 23 mg/g for the diffusive and sealed vessels respectively (Table 4.08).

**Table 4.08. Effects of different types of ventilation on chlorophyll and carotenoid contents based on fresh weight of leaves from 30 days old cauliflower cultures.**

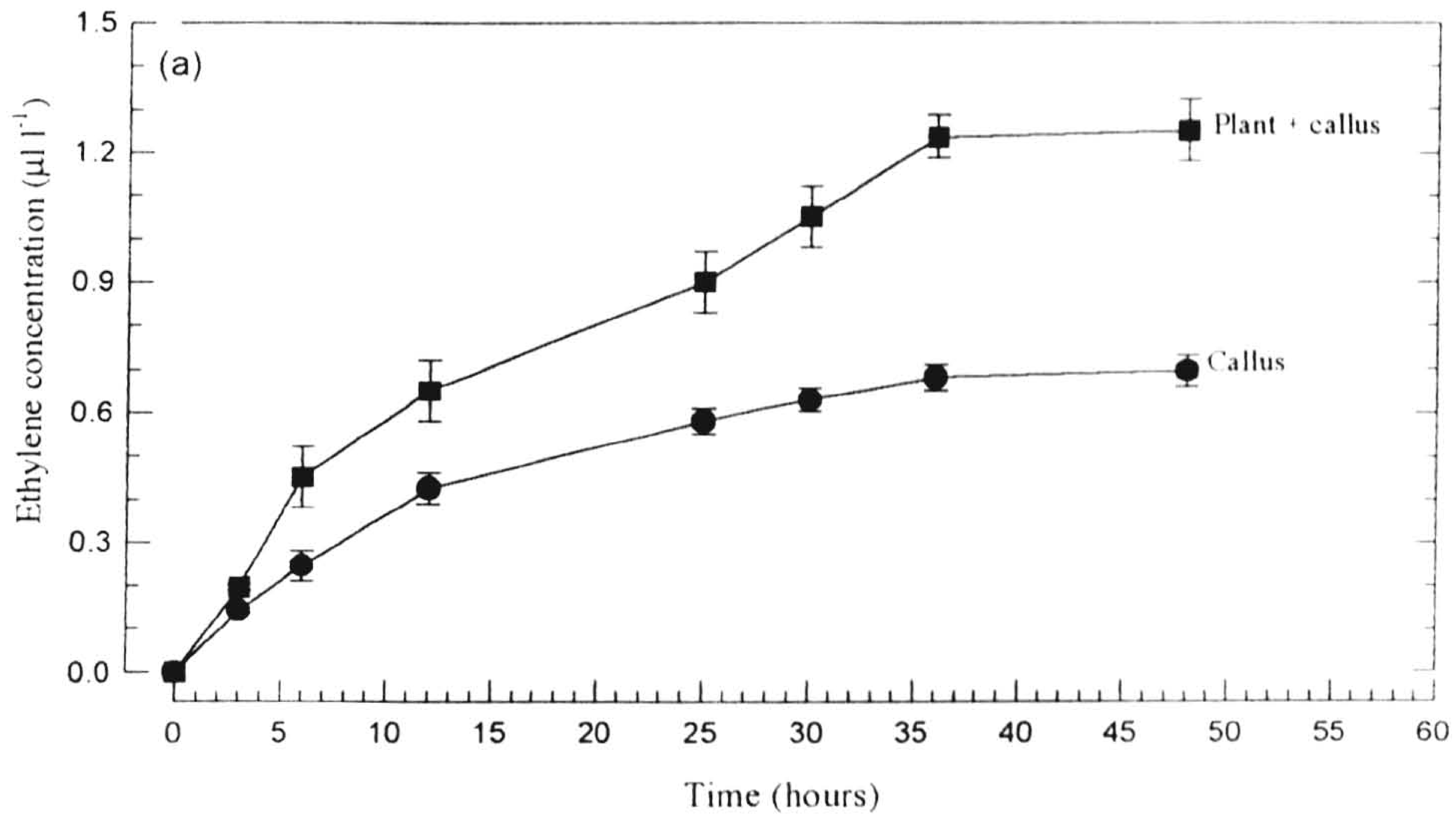
| Pigments | Sealed (with silicone rubber bung) | Diffusive ventilation (Capped with polypropylene) | Forced ventilation (Flow rate 3.5 $cm^3 min^{-1}$) |
|---|---|---|---|
| **Chlorophyll content** $\mu g (g\ fresh\ wt)^{-1}$ | 407.7±10.9 | 472.7±12.3 | 1342.7±45.2 |
| **Chl. a/b ratio** | 0.5±0.1 | 1.2±0.3 | 1.4±0.2 |
| **Carotenoid content** $\mu g (g\ fresh\ wt)^{-1}$ | 23.1±2.1 | 69.5±3.4 | 120.7±7.3 |

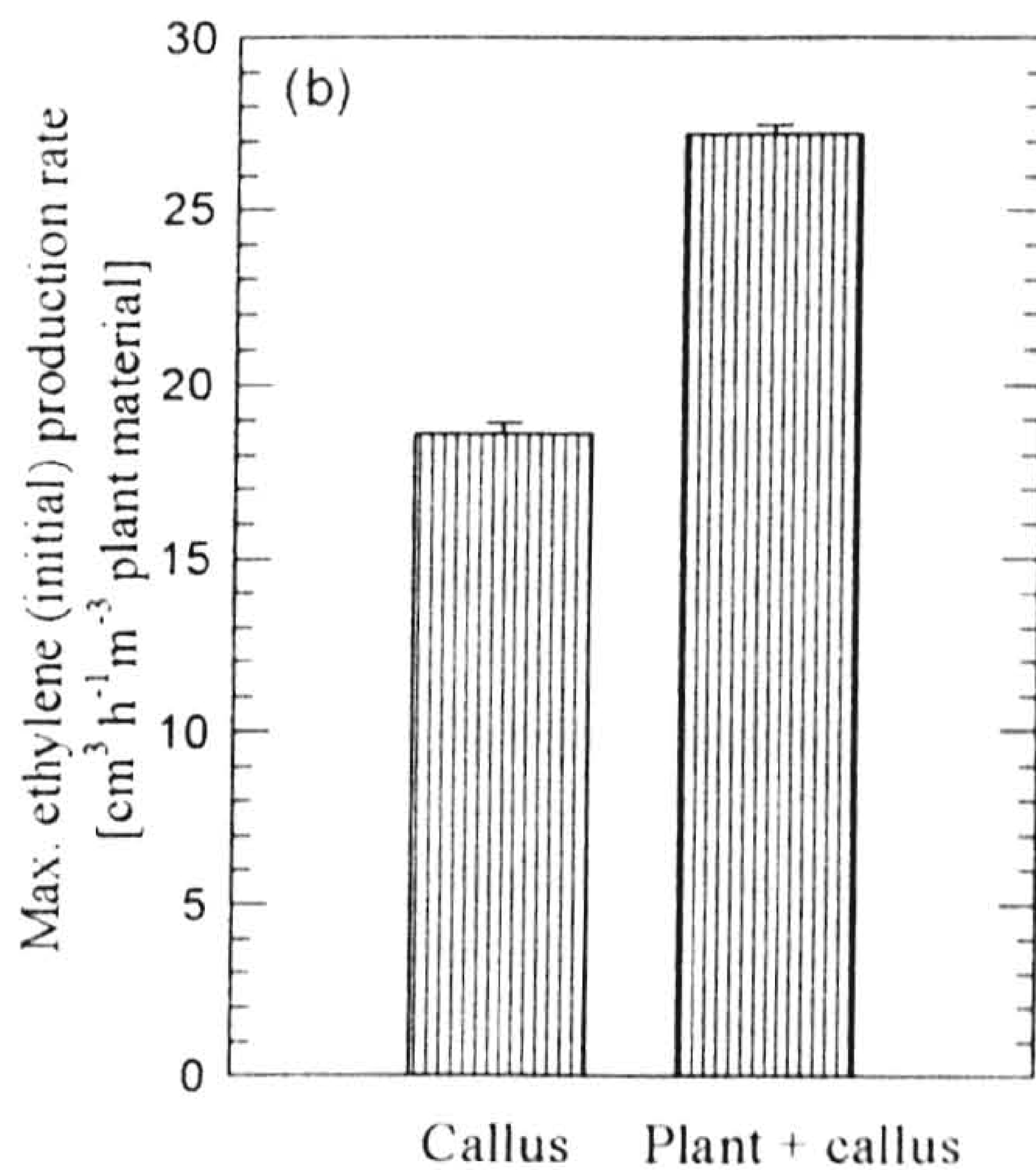
* Each value represents a mean ± SE of 5 replicates. Cultures were grown at *ca.* 25°C in continuous light; PAR = 150 $\mu mol\ m^{-2}s^{-1}$. 60 $cm^3$ culture vessels each contained one cutting.

### 4.3.5. Gaseous atmosphere of the culture vessels

#### 4.3.5.1. Ethylene

In sealed vessels very high ethylene concentrations (*ca.* 1.75 $\mu l\ l^{-1}$) were measured (Fig 4.08a,c) and extrapolating from data in the previous experiment it may be deduced that this was largely the cause of what was the poorest plant growth among the treatments (Table 4.06). The production rate of ethylene shown in Fig. 4.08 indicates that



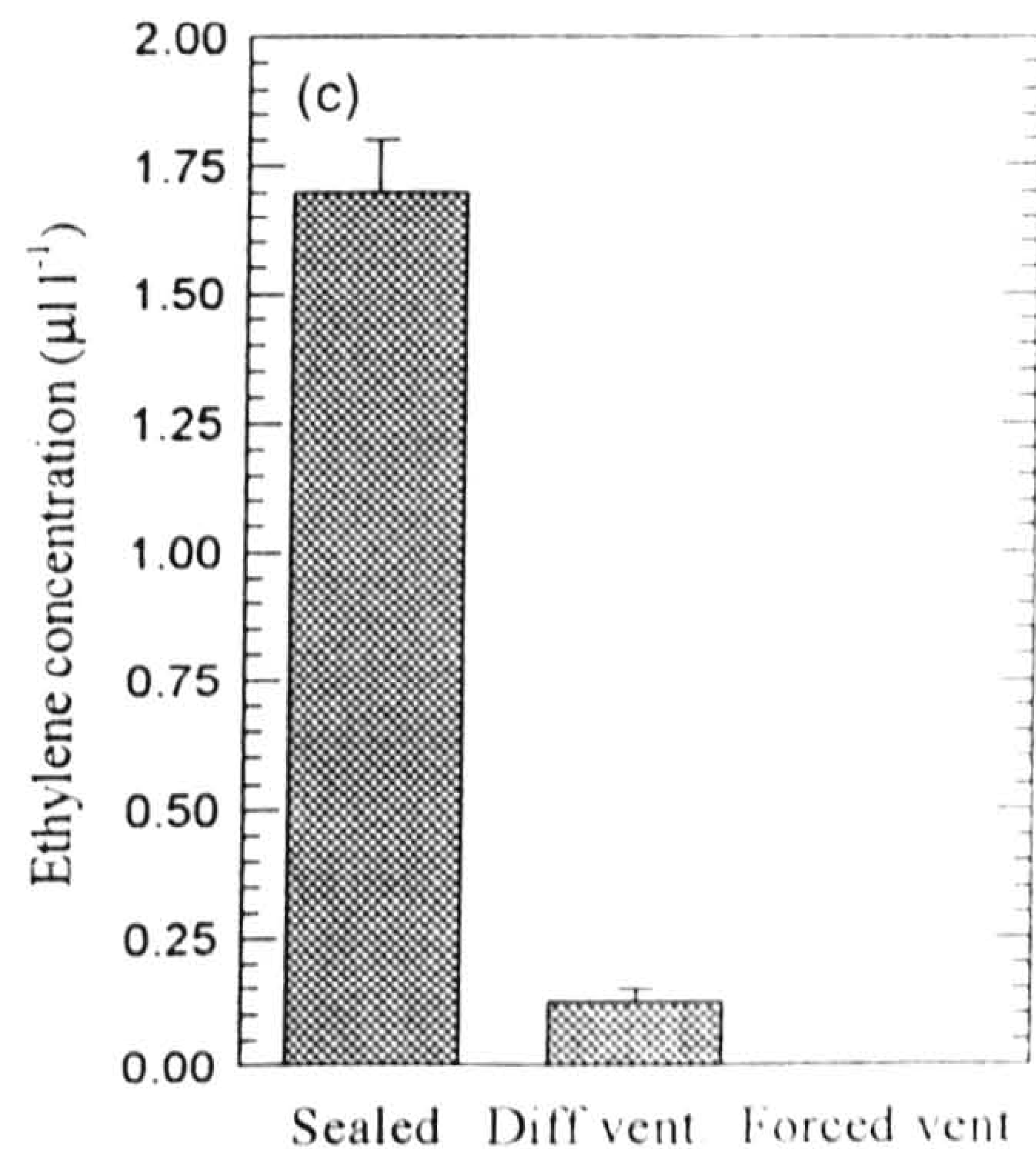




**Fig. 4.08.** **Cauliflower shoot culture (shoot + callus): ethylene concentrations in the culture head-spaces after 30 days (a) increases in ethylene concentration with time (on 30th day) in the sealed condition and (b) production rate of ethylene in the culture head-space in the sealed condition; (c) ethylene concentrations in the culture head-spaces with plants grown under different types of ventilation (sealed - with silicone rubber bung, diffusive - capped with polypropylene disc, fast forced ventilation - flow rate = 3.5 $cm^3$ $min^{-1}$). For (a) and (b) cultures had previously been exposed to the air for one hour and then resealed. 60 $cm^3$ culture vessels each contained one cutting, grown at *ca.* 25°C in continuous light; PAR = 150 μmol $m^{-2}s^{-1}$. Each symbol represents a mean ± SE of 5 replicates.**

approximately half of this ethylene was produced by the callus and half by the shoot system.

In culture vessels with forced ventilation the absence of ethylene was noted (Figs. 4.09c, 408,c). The better growth of cultures in this treatments suggests that the flushing out of ethylene from the culture vessels may have been necessary for normal growth, although as demonstrated earlier for the seedlings, $CO_2$ is likely to have been a most important supplementary cause of the better growth. Chi *et al.* (1990) reported on enhanced shoot regeneration from seedling explants of several *Brassica* genotypes in the presence of the ethylene inhibitors AVG and $AgNO_3$ in the nutrient medium. They also suggested that poor regeneration of cultured cells and tissues of *Brassica* may be attributed, at least partially, to ethylene produced by cultured plants. By using ethylene inhibitors and a number of recalcitrant *Brassica* genotypes, other authors showed that ethylene was one of the causes of recalcitrance (Chi and Pua 1989; Chi, Pua and Goh 1991; Lentini *et al.* 1988; Pua, Chi and Barfield 1991; Sethi, Basu and Guha-Mukherjee 1990).

#### 4.3.5.2. Carbon dioxide

The atmosphere in the head-space of the sealed vessels contained very high amounts of $CO_2$ despite the continuous illumination (Fig. 4.09b). At 30 days, this was *ca.* 5% being 215X that in vessels with forced ventilation, and 5.5X that in vessels with diffusive ventilation. Maximum $CO_2$ output into the culture vessels originated from the callus, and from the sealed containers the production rate of callus plus plantlet in air was 4600 $\mu M$ $m^{-3}$ callus $s^{-1}$ (Fig. 4.10) The plantlets also were net $CO_2$ contributors to the head-space (1737 $\mu M$ $m^{-3}$ plantlet $s^{-1}$) Concerning the callus *per se*, a similar result was reported by Adkins, Shiraishi and McComb (1989) who observed about 20% $CO_2$ in sealed petri-dish cultures of rice callus (IR42) after 20 days.

After 30 days the head-space of the diffusively ventilated culture vessels contained 0.9% (9000 $\mu l$ $l^{-1}$) of $CO_2$ (Fig. 4.09b), and in air the callus itself had a $CO_2$

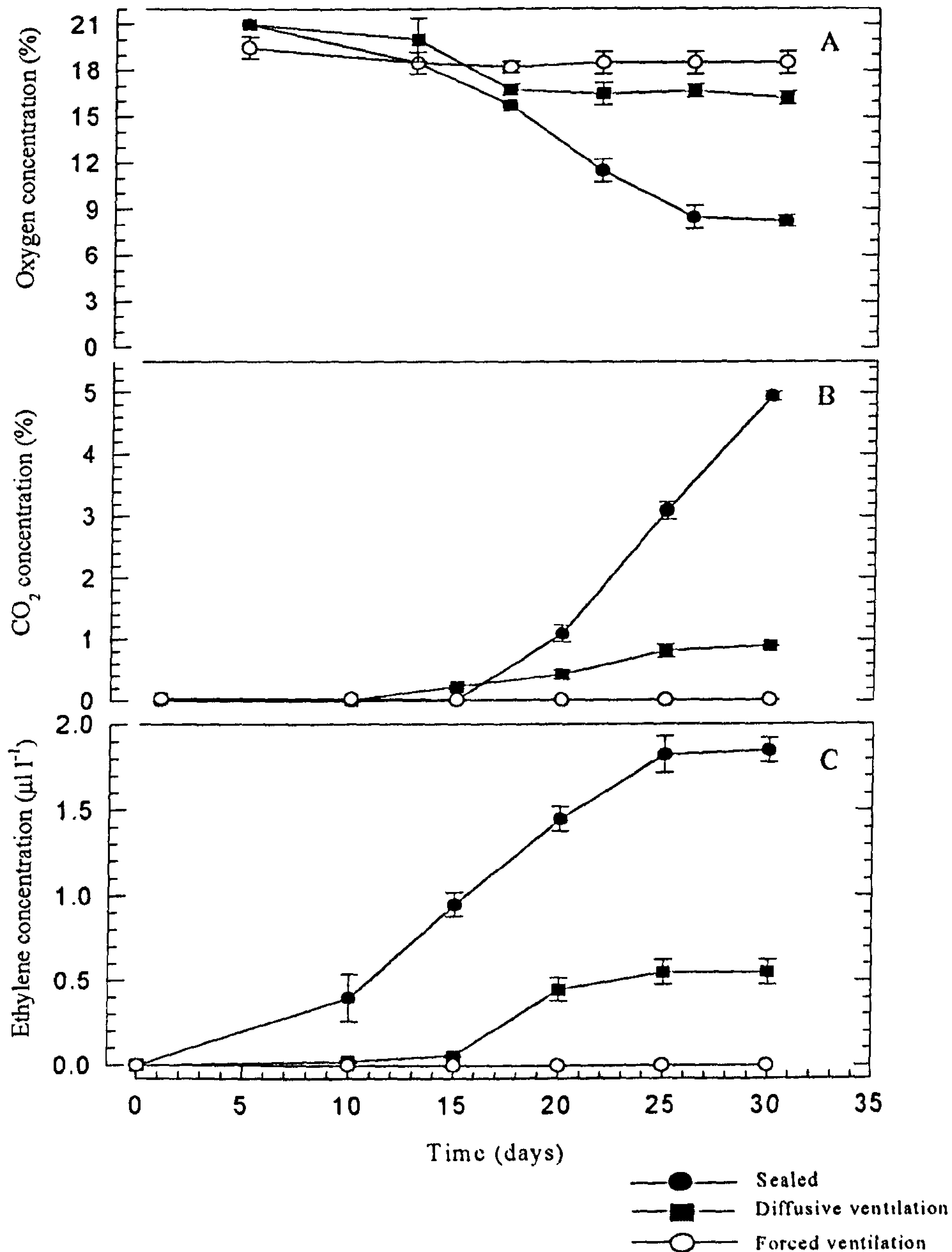



**Fig. 4.09.** Cauliflower shoot culture (shoot + callus): changes in (A) $O_2$ concentration, (B) $CO_2$ concentration and (C) ethylene concentration in the culture head-space with time. Cultures were grown under different types of ventilation (sealed - with silicone rubber bung, diffusive - capped with polypropylene disc, fast forced ventilation - flow rate = 3.5 $cm^3\ min^{-1}$). Culture vessel volume = 60 $cm^3$. Seedlings grown at *ca.* 25°C in continuous light; PAR = 150 $\mu mol\ m^{-2}s^{-1}$. Each symbol represents a mean ± SE of 5 replicates.

production rate of 836 μM $m^{-3}$ callus $s^{-1}$; the plantlets themselves were only just net $CO_2$ contributors (54 μM $m^{-3}$ plantlet $s^{-1}$). In forced ventilation plantlets were taking up $CO_2$ at the rate of 1464 μM $m^{-3}$ plantlet $s^{-1}$, showing that plantlets were photosynthesising (Fig. 4.10). The $CO_2$ production rate of the callus was 996 μM $m^{-3}$ callus $s^{-1}$ in this treatment. Similarly, high $CO_2$ levels under sealed conditions in the light, have been reported for various species, for example with *Prunus* (Righetti *et al.* 1990), *Pinus radiata* (Kumar, Reid and Thorpe 1987) and *Actinidia deliciosa* (Infante, Magnanini and Righetti 1989).

It should be noted that $CO_2$ production or uptake might have been different at the working levels of oxygen in the vessels, particularly in the sealed vessels. However, this possibility was not explored during this study.

#### 4.3.5.3. Oxygen

During the course of the experiment oxygen concentrations in the sealed and diffusively ventilated vessels fell as $CO_2$ levels rose. With FF-ventilation, on the other hand, oxygen levels remained fairly constant at levels a little below atmospheric. In the sealed vessels, the oxygen concentrations eventually were reduced to only 7.1% after 30 days of culture compared to 15.1% and 19.2% in the diffusive- and FF- ventilation types respectively (Fig. 4.09a). It is evident that the major decline in oxygen did not take place until after 10 days of treatment, and in the sealed case probably reflects the onset of vitrification of the plantlets in response to the higher levels of ethylene accumulation (Fig. 4.09c). It is possible that the lowered oxygen concentrations in the sealed system might have been accompanied by some anoxic core development in the calli; such an effect could have contributed to the higher $CO_2$ levels found in this treatment by initiating anaerobic respiration. In any future study it would be interesting to test for anaerobic by-products in sealed systems with calli.

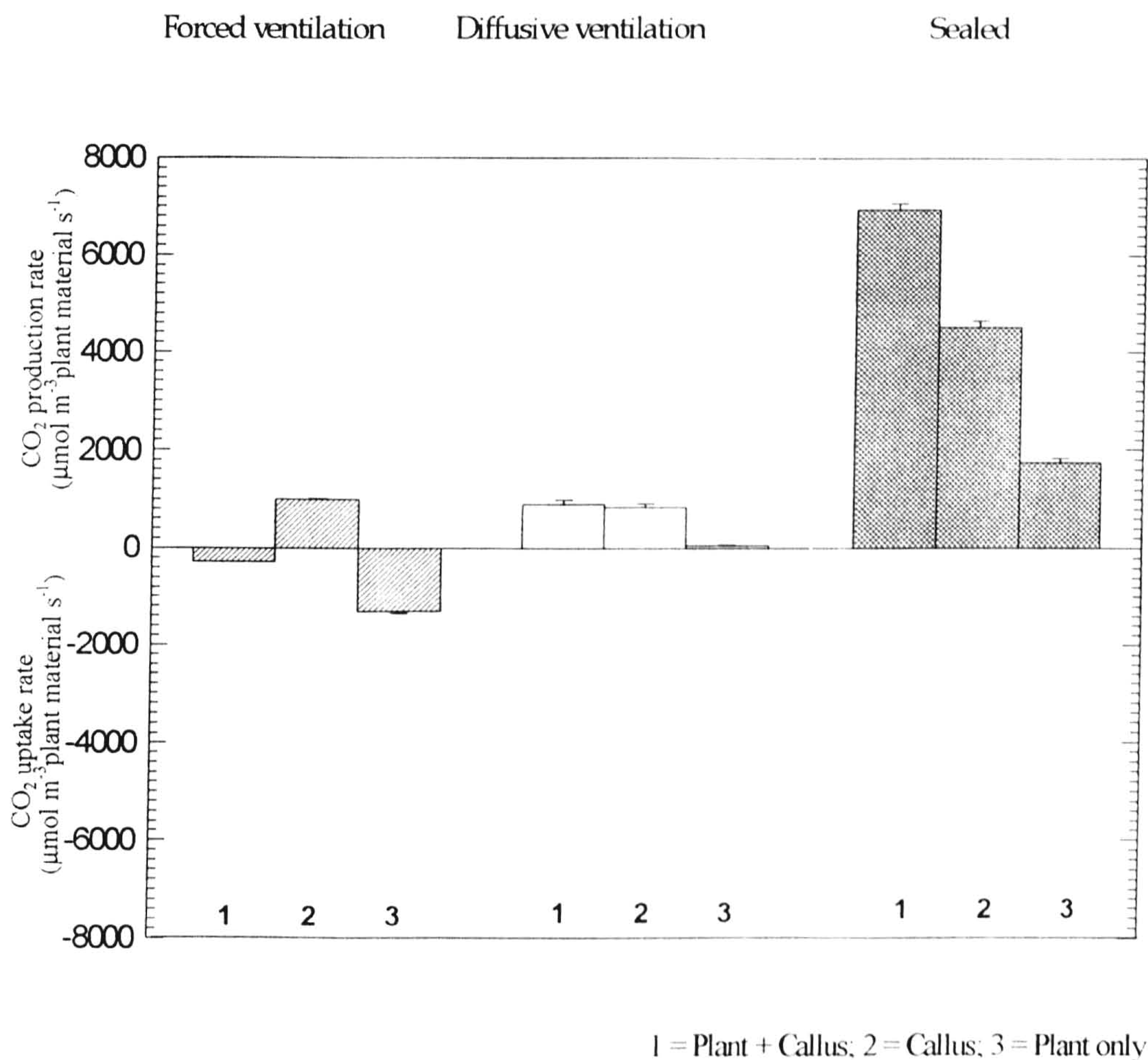



**Fig. 4.10.** **Cauliflower shoot culture (shoot + callus; 30 days old): effects of different types of ventilation on carbon dioxide production/uptake rate. Cultures had been previously grown under sealed (silicone rubber bung), diffusive ventilation (polypropylene disc) and fast forced ventilation condition (flow rate=3.5 $cm^3$ $min^{-1}$). 60 $cm^3$ culture vessels each contained one cutting, grown at *ca.* 25$^oC$ in continuous light; PAR = 150 µmol $m^{-2}s^{-1}$. Each symbol represents a mean ± SE of 5 replicates.**

The results are consistent with the recent findings of some other authors. In tightly sealed vessels with *Ficus* plantlets alone, oxygen concentrations of approximately 10% were observed (Jackson *et al.* 1991), while in sealed petri-dishes containing rice callus the oxygen concentration had declined to between 2 and 5% after 24 days of culture (Adkins, Shiraishi and McComb 1990).

## 4.4. FINAL COMMENTS

The results indicate that in cauliflower seedlings the sealing of culture vessels can have serious inhibitory effects on growth and development and photosynthesis, induce leaf epinasty and vitrification and reduce leaf chlorophyll and carotenoid contents. To smaller extents these effects, which are associated with ethylene accumulation, were seen with both diffusive and slow flow ventilation, but they can be overcome *to some extent* in all these systems by the use of ethylene inhibitors, the best effects being with 10 μM $AgNO_3$. It seems reasonable to conclude, therefore, that these adverse effects were at least partly due to ethylene accumulation in the head-spaces of the culture vessels.

However, with the seedlings, the use of the fast-flow ventilation system *per se*, (3.5 $cm^3$ $min^{-1}$) and without any ethylene inhibitor, produced even better growth, rates of photosynthesis, chlorophyll contents, than did the use of silver nitrate with the sealed condition or with diffusive or slow flow ventilation. This could have been due not only to the complete flushing out of ethylene by the fast flow system, but also to the additional supply of $CO_2$.

Similarly good rates of growth were also produced with shoot culture in the fast flow ventilation, and without the use of ethylene inhibitors. The removal of ethylene by this system would be expected to be particularly beneficial to shoot culture, where callus can be an important source of the gas (see also Chapter VIII).

# CHAPTER V

## EFFECTS OF CLOSED SYSTEM, DIFFUSIVE AND FORCED VENTILATION ON THE GROWTH AND PHYSIOLOGY OF TOBACCO *IN VITRO*

### 5.1. INTRODUCTION

To prevent microbial contamination and to reduce desiccation, tissue cultures vessels conventionally are protected in various ways e.g. with cotton-wool bungs, screw caps, polypropylene membranes etc. Under these protective conditions an exchange of gases between the tissues and the external atmosphere is limited; consequently the gaseous composition of the culture vessel may alter and differ considerably from that of the atmosphere *e.g.* in terms of water vapour, oxygen, carbon dioxide etc. (Buddendorf-Joosten and Woltering 1994). Depending on the type of tissue and species the culture itself may also produce various types of gases e.g. ethylene (Huxter, Reid and Thorpe 1979; Adkins, Shiraishi and McComb 1990).

It is well known that tobacco callus produces ethylene and that the actual quantity depends upon factors such as light and the age of the callus (Huxter, Reid and Thorpe 1979). However, the effects of ethylene on *in vitro* tobacco culture are diverse. Horner *et al.* (1977) found that the removal of ethylene by charcoal had no marked effect on plantlet formation from cultures of tobacco anther. Huxter, Thorpe and Reid (1981) demonstrated that a low concentration of ethylene speeds up the rate of shoot emergence in tobacco, but higher concentrations have the opposite effect. Earlier in 1979, Huxter, Reid and Thorpe showed that a mercuric perchlorate sink (for the absorption of ethylene) had no significant effect on the growth of tobacco callus; however, he established that there was a strong positive correlation between ethylene production and growth rate. On the other hand, Bolton and Freebairn (1975) showed that large doses of exogenous ethylene inhibit tobacco callus growth.

Very few studies have been concerned with the role of elevated $CO_2$ concentrations on tobacco plant growth and development. Mousseau (1986) enhanced the $CO_2$ concentration from 450 to 900 $\mu l\ l^{-1}$ and reported positive effects on the growth of all parts especially the roots. Solárová *et al.* (1989) revealed that an increase in $CO_2$ concentration to 10 or 40 g $m^{-3}$ (10 g $m^{-3}$ = 7608 $\mu l\ l^{-1}$; 40 g $m^{-3}$ = 30435 $\mu l\ l^{-1}$)* caused increases in plantlet growth rate, dry matter accumulation (especially in stems and roots), and leaf area and thickness.

The aim of the work described in this chapter was to determine the effects of various methods of ventilation on the growth and development of *in vitro*-grown tobacco seedlings, particularly with a view to establishing whether endogenous ethylene has a significant effect on growth and whether $CO_2$ supply with conventional capping systems can be limiting. To this end, the effects of ethylene inhibitors and precursors were investigated, as were the effects of enhancing $CO_2$ concentrations in the culture vessels. A major part of the investigation was to find ways of improving culture growth and development by introducing diffusive and forced ventilation into the vessels and by comparing plantlet growth with that achieved in sealed vessels.

* Atmos. $CO_2$ = 0.46 g $m^{-3}$ = 350 ppm ($\mu l\ l^{-1}$)

## 5.2. MATERIALS AND METHODS

### 5.2.1. Plant material and sterilization

Seeds of tobacco (*Nicotiana tabacum* cv. White burley) were surface-sterilized by immersing in sodium hypochlorite solution (2% w:v) for 3 min, then rinsed three times with sterile water and sown at a density of 4 seeds per tube on to an MS medium in culture vessels as described in Chapter IV. Germination usually occurred within 5-7 days. Unless stated otherwise, the plants were grown under continuous light at a growth room temperature of 25°C and PAR of 150 μmol $m^{-2}$ $s^{-1}$. Relative humidity was usually *ca.* 30-35% but since there was no humidistat it could vary from < 20% to *ca.* 50%.

Unless stated otherwise, culture vessel size and volume, media preparation, and methods of measuring ethylene, carbon dioxide, oxygen, chlorophyll and carotenoid contents and photosynthetic rate etc., were as described in Chapter IV. The basal medium used was MS (Murashige and Skoog 1962) with various additions as described below. Stocks were filter sterilized (Millipore, 0.22 μm) and added where necessary in the medium (full strength MS) after autoclaving.

### 5.2.2. Experiments

#### 5.2.2.1. Effects on growth and ethylene levels of the presence or absence of various ethylene inhibitors and precursors in closed vessels

Silver nitrate ($AgNO_3$ - 2.5, 5, 10 and 20 μM) and cobalt chloride ($CoCl_2$- 5, 10 and 20 μM) as ethylene inhibitors and ACC (5, 10 and 20 μM) as an ethylene precursor, were used at different concentrations in the rooting medium (Table 5.01); also, $AgNO_3$ (5 μM) and ACC (5 μM) were used in combination. Stock solutions were prepared and stored as described in Appendix II.

Surface sterilized seeds were inoculated on to the culture tubes containing ½ strength MS medium, sealed (Si-rubber bung) and transferred to the growth room. Ethylene concentrations in the head-spaces of the culture vessels were measured (5 replicates per treatment) on the 10th day and then harvested. Growth measurements

included leaf number, fresh weight and area, stem fresh weight, and root number and maximum length.

#### 5.2.2.2. Growth and development of seedlings: the evaluation of the closed, diffusive and forced ventilation systems with and without $AgNO_3$ or ACC in the culture medium

The silver nitrate and ACC were added to the medium (half strength MS with 3% sucrose), as necessary, after filter sterilization (Millipore, 0.22 μm). Four seeds were inoculated per culture vessel (replicate) and the cultures were transferred to the growth room.

In all, 16 treatments were examined with five replicates per treatment as follows:
(a) controls without additives to ½ strength MS medium and with four types of ventilation:

(i) sealed with silicone rubber bung,

(ii) capped with polypropylene to give diffusive ventilation,

(iii) capped with slow flow (SF) HIC ventilation apparatus (1.5 $cm^3$ $min^{-1}$), and

(iv) capped with fast flow (FF) HIC ventilation apparatus (3.5 $cm^3$ $min^{-1}$).

(b) ½ strength MS medium with 2.5 μM $AgNO_3$ plus ventilation types (I) - (iv) as above.

(c) ½ strength MS medium with 2.5 μM ACC plus ventilation types (i) - (iv) as above.

Ethylene and $CO_2$ concentrations in the culture head-space and photosynthetic rates were measured on days 9 - 10; growth and chlorophyll contents were measured on days 10 - 11.

Photosynthetic rates were measured as required by the methods described in Chapter IV (Section 4.2.4.5).

#### 5.2.2.3. Growth and development of seedlings: effects of elevated levels of $CO_2$ and ethylene

Culture vessels, each capped with a polypropylene membrane and containing four 7 day-old seedlings growing in half strength MS medium, were exposed to various

concentrations of $CO_2$ and / or ethylene by enclosing them in 5-litre glass chambers (desiccators without the desiccant) with five vessels per chamber and five replicates/treatment (Fig. 5.01). The chambers' atmospheres were adjusted every 12 h for the first 5 days, and every 4-6 hours thereafter, to be as follows:

(1) 3000 $\mu l\ l^{-1}$ $CO_2$; (2) 1500 $\mu l\ l^{-1}$ $CO_2$; (3) 350 $\mu l\ l^{-1}$ $CO_2$; (4) 350 $\mu l\ l^{-1}$ $CO_2$ + 1.5 - 2.0 $\mu l\ l^{-1}$ ethylene; (5) 350 $\mu l\ l^{-1}$ $CO_2$ + 15 $\mu l\ l^{-1}$ ethylene; (6) 0 - 5 $\mu l\ l^{-1}$ $CO_2$.

To maintain the lowest $CO_2$ concentration (0 - 5 $\mu l\ l^{-1}$), a glass vial containing (50 $cm^3$) of soda lime was placed in the chamber To enrich the $CO_2$ concentration (1500 and 3000 $\mu l\ l^{-1}$), the chambers were supplied with air mixed with $CO_2$ from a gas cylinder. For atmospheric levels (350 $\mu l\ l^{-1}$ $CO_2$) air was supplied from a compressed air cylinder every 3 days. The ethylene : air mixture (350 $\mu l\ l^{-1}$ $CO_2$ + 1.5 or 15 $\mu l\ l^{-1}$ ethylene) was created by injecting appropriate volumes of ethylene-enriched air (22.9 $\mu l\ l^{-1}$) from a gas-cylinder (Fig. 5.01). Cultures were maintained in growth room conditions for 15 days.

The carbon dioxide levels were monitored by means of IRGA analysis, and ethylene by GC, as described in Chapter IV.

#### 5.2.2.4. Shoot culture from cutting: the effects of closed, diffusive and forced ventilation systems

For direct shoot regeneration, shoot tips of 7 days old seedlings were used. Explants were inoculated on full strength MS medium supplemented with BAP (0.5 mg $l^{-1}$) + NAA (0.1 mg $l^{-1}$) for adventitious shoot induction. Vessels were capped with one of the following: (a) silicone rubber bung, (b) polypropylene membrane, (c) SF ventilation apparatus (flow = 1.5 $cm^3\ min^{-1}$); (d) FF ventilation apparatus (flow = 3.5 $cm^3\ min^{-1}$). The cultures were incubated under growth room conditions.

Concentrations of $CO_2$, $O_2$ and ethylene were measured on the 30th day. $CO_2$ production rate for plant + callus, were measured in the light on the 30th day. For

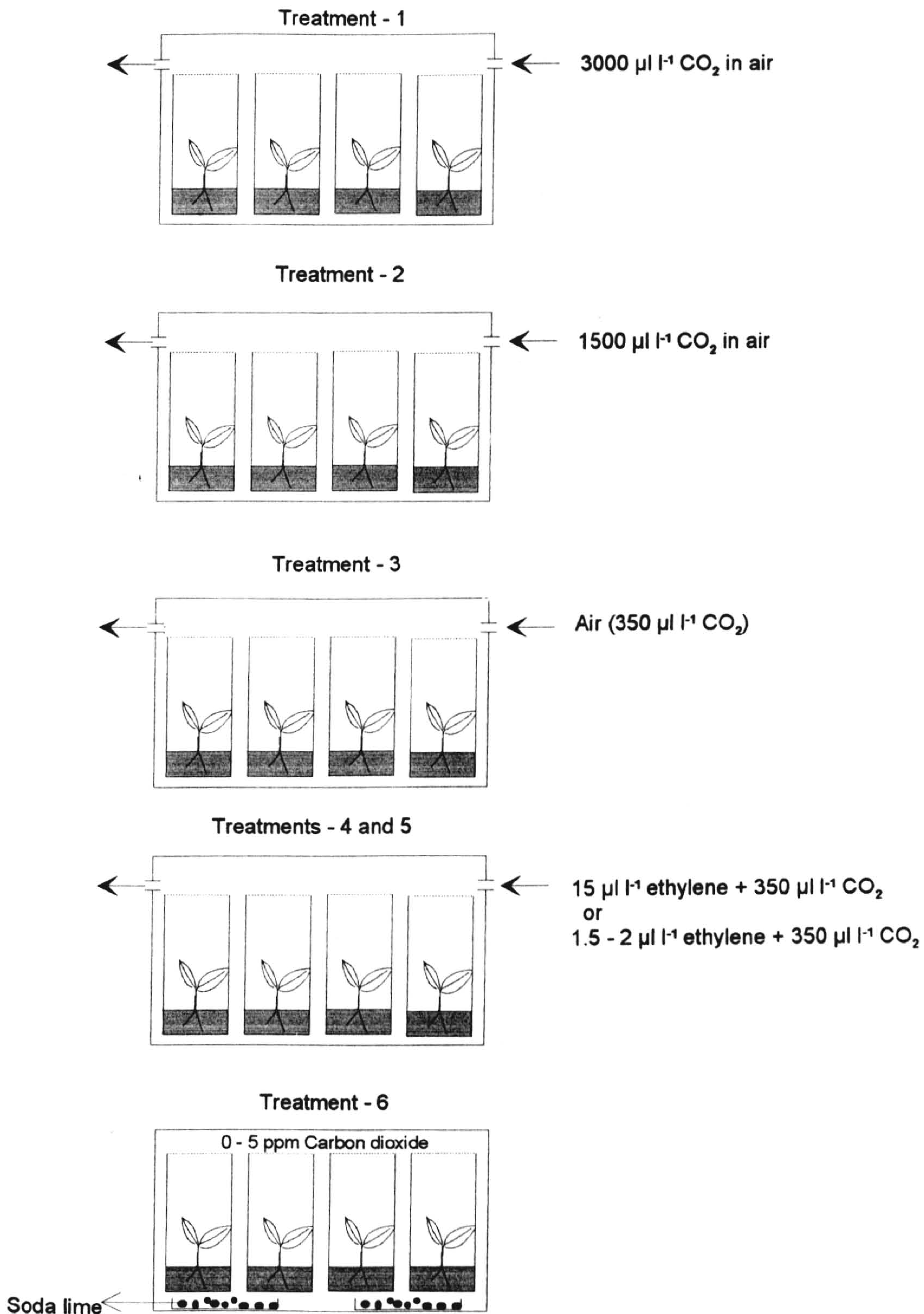



Fig. 5.01. Showing the assembly for assessing the effects of enhanced $CO_2$ ± ethylene on tobacco seedling growth. Note that the drawings are not to scale and chamber volume was 5000 ml and culture vessel volume 60 ml. The chambers were recharged at intervals with the appropriate gas mixtures indicated and the chambers were then re-sealed. The culture vessels were capped with polypropylene membranes.

measuring $CO_2$, production rates the vessels were first charged with air. Growth measurements of plants and callus were measured on the 31st day.

### 5.2.2.5. Growth, chlorophyll contents and yellowing of leaves: the effects of closed, diffusive and forced ventilation systems

Shoot tips derived from 15-day old seedlings were base-inserted on hormone-free ½ - strength MS medium in vessels each capped with a FF-ventilation apparatus (3.5 $cm^3$ $min^{-1}$). After 7 days of culture (FW approx. = 100 mg; leaf area = approx. 5.5 $cm^2$), the FF-ventilation apparatus was replaced with the systems described in 5.2.2.4.

Starting with 20 - 30 plants at the beginning of the experiment, harvesting proceeded throughout the experiment (days 1 - 15), by removing 2 - 3 plants each time for analysis of growth and chlorophyll contents. Measurements of ethylene and $CO_2$ concentrations in the head-spaces (5 replicates per treatment) were performed on a daily or alternate daily basis.

### 5.2.2.6. Continuous exposure to ethylene under FF-ventilation

As described in the previous experiment, plantlets were cultured on MS medium (½ strength) in vessels fitted with the FF-ventilation apparatus. After 15-days when plantlet fresh weight was approx. 350 mg / plantlet, four vessels, with one plantlet per vessel, were transferred to continuous ethylene exposure by inserting the membrane ends of the FF-units into a stream of ethylene-enriched air (1.5 - 2.0 $\mu l\ l^{-1}$) in a 'wind-tunnel' (Fig. 5.02); four vessels remained untreated as controls. The plants were harvested on the fifth day and the fresh weights, leaf numbers, chlorophyll content etc., were recorded.

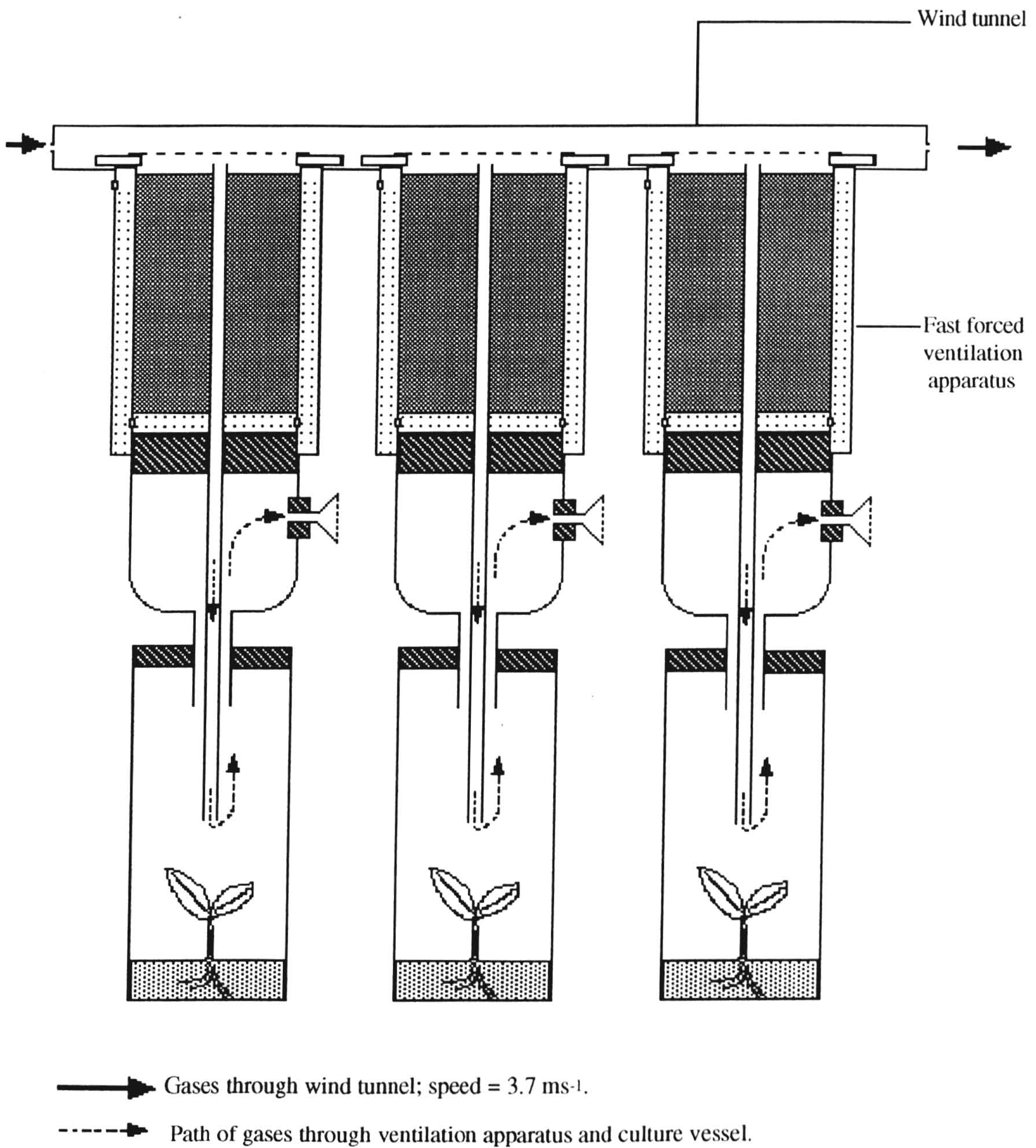



**Fig. 5.02. The system for continuous ethylene (1.5 - 2.0 μl $l^{-1}$) exposure under fast forced ventilation (> 5.0 $cm^3$ $min^{-1}$) in the culture atmosphere of *in vitro*-grown tobacco plantlets. For details of fast forced ventilation apparatus see Fig. 2.16.**

## 5.3. RESULTS AND DISCUSSION

### 5.3.1. Effects of ethylene inhibitors and precursors on the growth and development of tobacco seedlings under closed system ventilation

#### A. Silver nitrate and cobalt chloride

The results from using these ethylene inhibitors did not show any clear evidence that endogenous ethylene production might be an inhibitor of growth in sealed conditions (Table 5.01). The silver may have marginally stimulated leaf growth at the lowest concentrations (2 and 5 μM Ag) but was apparently the cause of some growth inhibition at the higher concentrations (10 and 20 μM Ag). However, at these low silver nitrate concentrations the colour of the leaves remained fully green, whereas in the additive-free controls some of the leaves became yellowish. Cobalt may have stimulated leaf growth at each concentration and root numbers were generally increased. Root extension may have been enhanced by the silver.

The ethylene concentrations in the head-spaces of the vessels were relatively low in all treatments (Fig. 5 03); this was probably due to the small size of seedlings. However, in the controls and in the presence of silver nitrate, ethylene levels were substantially more than in the presence of cobalt. Again, as described in Chapter IV, these findings are in agreement with those of Reid and Bradford (1984), who observed that the presence of cobalt can stop ethylene production, whereas silver simply inhibits its biological action. In the control vessels the ethylene had accumulated to a concentration of 0.31 $\mu l\ l^{-1}$ after 10 days and this was five times that of the cobalt chloride treatment (5μM).

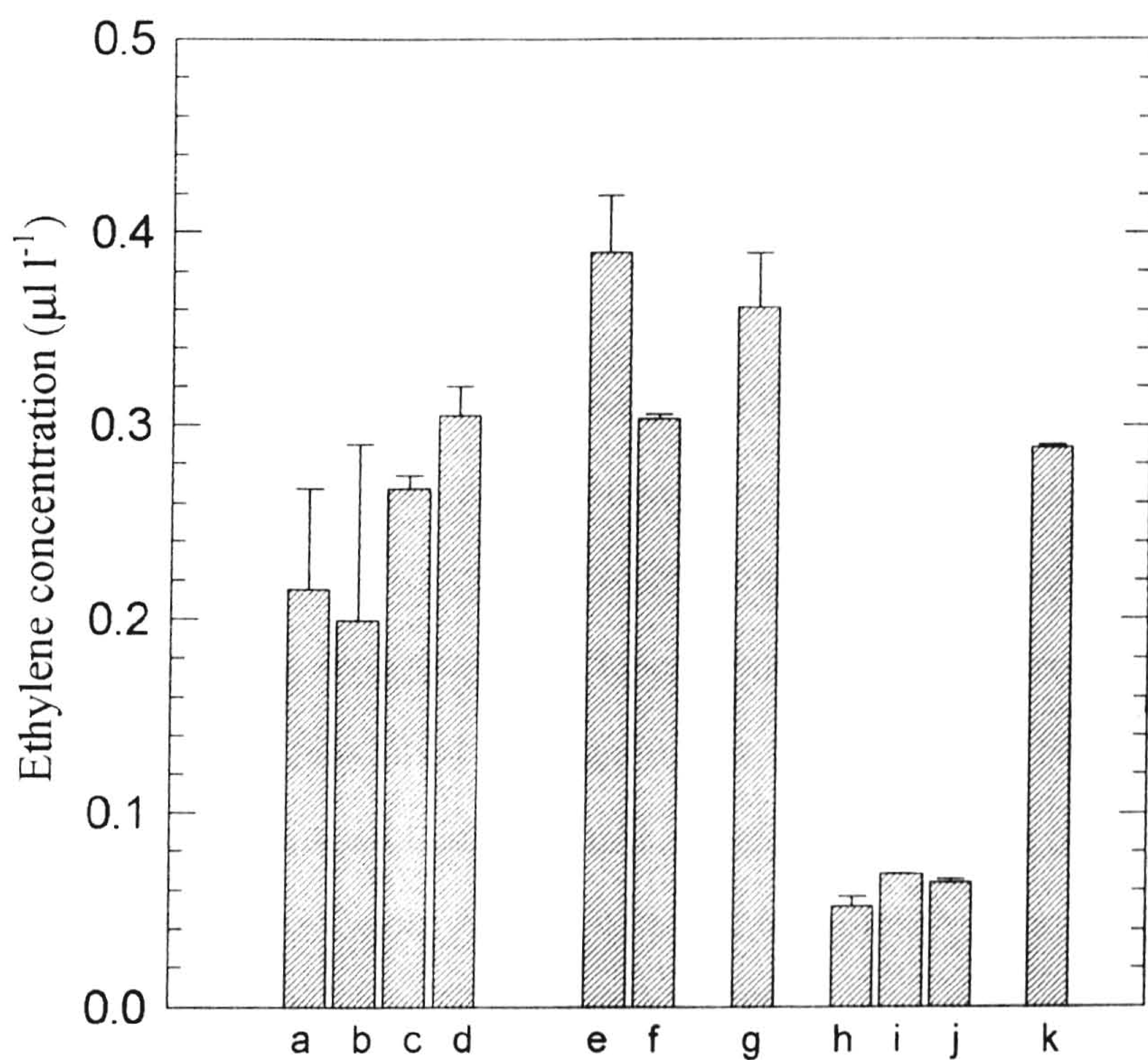
#### B. ACC

The addition of the ethylene precursor (ACC) *per se* to the medium inhibited growth and development of seedlings, particularly in terms of the area and fresh weights of leaves. At both concentrations the latter were approx. half of those of the controls. Nevertheless, only at the higher concentration of ACC was the ethylene concentration in the head-space significantly higher (1.3X) than that of the control (Fig. 5.03). It is not

**TABLE 5.01.** **Effects of ethylene inhibitors and precursor on the growth and development of tobacco seedlings.**

| Treatments | Leaves | | | Stem | Root | |
|---|---|---|---|---|---|---|
| | No | Area ($cm^2$) | Fresh weight (mg) | Fresh weight (mg) | No | †Max. Length (mm) |
| Control | 6.2±0.8 | 0.9±0.04 | 17.2±0.8 | 2.5±0.1 | 3.6±0.07 | 9.9±0.3 |
| $AgNO_3$ (20μM) | 3.7±0.2 | 0.73±0.2 | 11.3±1.9 | 2.4±0.2 | 3.3±0.09 | 12.2±3.1 |
| $AgNO_3$ (10μM) | 3.9±0.4 | 0.8±0.5 | 16.9±2.1 | 2.2±0.3 | 2.3±0.03 | 11.6±1.1 |
| $AgNO_3$ (5μM) | 4.9±0.7 | 1.1±0.3 | 18.7±2.1 | 2.5±0.6 | 1.8±0.7 | 10.7±1.3 |
| $AgNO_3$ (2μM) | 7.5±0.3 | 1.2±0.6 | 18.9±0.7 | 2.7±0.5 | 2.5±0.7 | 10.9±1.0 |
| $CoCl_2$ (20μM) | 6.1±0.9 | 1.1±0.1 | 17.9±0.3 | 2.4±0.4 | 4.1±0.3 | 8.1±0.4 |
| $CoCl_2$ (10μM) | 6.9±0.2 | 0.97±0.1 | 18.3±0.6 | 2.4±0.3 | 4.3±0.2 | 10.1±0.7 |
| $CoCl_2$ (5μM) | 7.2±0.7 | 0.99±0.2 | 18.4±0.7 | 2.5±0.1 | 4.5±0.7 | 10.9±0.5 |
| ACC (10μM) | 5.3±0.2 | 0.45±0.1 | 8.2±0.7 | 2.3±0.8 | 3.3±0.5 | 3.9±0.6 |
| ACC (5μM) | 6.1±0.1 | 0.46±0.2 | 9.1±0.3 | 2.4±0.4 | 4.1±0.3 | 6.8±1.0 |
| ACC (5μM) + $AgNO_3$ (5μM) | 6.9±0.2 | 0.67±0.3 | 9.9±0.3 | 2.7±0.4 | 5.7±0.6 | 6.7±0.5 |

* 10 days old seedlings; 60 ml glass containers were sealed with silicone rubber bung; each value represents a mean ± SE of 20 seedlings; †mean maximum root length (3 roots from each seedling). Seedlings grown at *ca.* 25°C in continuous light ( PAR = 150 μmol $m^{-2}s^{-1}$).




[a = $AgNO_3$ (20 µM); b = $AgNO_3$ (10 µM); c = $AgNO_3$ (5 µM); d = $AgNO_3$ (2.0 µM); e = ACC (10 µM); f = ACC (5 µM); g = ACC (5 µM)+$AgNO_3$ (5 µM); h = $CoCl_2$ (20 µM); i = $CoCl_2$ (10 µM); j = $CoCl_2$ (5 µM) and k = control - no additives].

**Fig. 5.03. Effects of ethylene inhibitors and precursor in the rooting medium (MS - ½ strength) on ethylene concentration of the culture atmosphere of 10 days old tobacco seedlings grown in sealed condition; each bar represents a mean ± SE of 4-5 replicates.**

possible, therefore, to correlate the inhibitory effect of the lower of the two ACC concentrations directly with higher ethylene levels; however, it might be that the ACC did produce higher concentrations of endogenous ethylene in this treatment than were found in the controls. The combination of 5 μM $AgNO_3$ and 5 μM ACC restored leaf growth to the same as that of the control, although increasing the ethylene accumulation in the medium; this again pointed to some ethylene involvement in the growth of tobacco seedlings

A major difficulty with this whole experiment was the poor growth made in these closed treatments which, as later treatments showed, may have been due as much to $CO_2$ limitation as to any ethylene effects. However, although the inhibitor treatments *per se* did not give very clear evidence for ethylene inhibition in sealed vessels, the ACC additions and the ACC plus silver do suggest that ethylene could be important. One of the difficulties may have been that the 2 μM silver was too high a concentration. Much later a trial was conducted using Ag at 0.01 and 0.06 μmol $l^{-1}$; here it was found that at an early stage (5 days) the plants in the 0.06 μmol $l^{-1}$ Ag treatment were noticeably larger than the controls (data not shown). Unfortunately, however, the better growth was not sustained and eventually there was little difference between the silver treatments and the controls. It may be that, if such low levels of silver are required to inhibit ethylene action and at the same time avoid silver toxicity, the silver activity in the medium may have been only short-lived: the silver in $AgNO_3$ is a readily precipitated ion, especially in the light or in contact with organic matter. In the literature, however, there are some reports of tobacco being unresponsive to ethylene absorbers. For example Huxter, Reid and Thorpe (1979), showed that the use of mercuric perchlorate as an ethylene absorber had no significant effect on the growth of tobacco callus. Similarly Horner *et al.* (1977) found that the ethylene effect on adrogenesis in tobacco anther culture was unaffected by removal of ethylene from the gas phase by charcoal. In the present study the beneficial effects of silver on cauliflower (Chapter IV) were more obvious than on tobacco.

Cauliflower is less sensitive to high concentrations of silver and hence higher concentrations were used; the silver ion activity in the medium might, therefore, have been sustained for longer. Further evidence that endogenous ethylene might be a problem when tobacco is grown with restricted ventilation is provided by the data from other experiments reported below.

Although the results for cobalt were somewhat better than for silver, cobalt was not used in subsequent experiments. This was for the reason that since it inhibits ethylene production, and since at least some ethylene is thought to be required for normal growth, it might be inappropriate to continue employing it.

### 5.3.2. Growth and development of seedlings : the effects of closed, diffusive and forced ventilation systems with and without $AgNO_3$ or ACC in the culture medium

#### 5.3.2.1. Growth (no additives)

By far the best growth was observed in FF-forced ventilation (3.5 $cm^3$ $min^{-1}$) and the poorest was in the sealed vessels (Table 5.02; Plate 5.01).

In the FF-system the leaf area and fresh weights were respectively 10X and 16X those of the sealed controls; however, the numbers of leaves were only marginally higher. The diameters of the stems and their lengths were 4.1X and 3.1X those of the sealed treatments and stem fresh weight (20.1±2.0 mg) and root numbers (12±1.5) in the FF-system were respectively >15X and >3X those of the controls. Similarly, root lengths were also appreciably higher, being 3.6X those of the sealed vessels. Also, it should be noted that in the sealed vessels the leaves became pale yellow in colour, whereas in forced ventilation they remained a dark green. Furthermore, in the sealed condition the leaves showed epinastic curvature and the roots sometimes grew out of the medium.

Plants grown with diffusive or SF-ventilation achieved growth which was intermediate between that of the FF-ventilation and sealed treatments. In all aspects, however, plants grew better with the SF-ventilation system than with the diffusive system (see Table 5.02). Even so, growth was much better with FF-ventilation· stem

**TABLE 5.02.** **Effects of different types of ventilation, ACC and $AgNO_3$ on the growth and development of tobacco seedlings.**

| Treatments | | Leaves | | | Stem | | | Root | |
|---|---|---|---|---|---|---|---|---|---|
| | | No | Area ($cm^2$) | Fresh weight (mg) | Diameter (mm) | Length (mm) | Fresh weight (mg) | No | †Max. Length (mm) |
| **Control** | | | | | | | | | |
| | Sealed | 5.3±0.9 | 0.67±0.2 | 10.6±0.7 | 0.47±.02 | 2.2±0.7 | 1.3±.03 | 3.4±0.3 | 11.5±0.5 |
| | Diff. vent. | 7.8±0.3 | 1.3±0.5 | 55.1±3.9 | 0.56±.09 | 2.9±.9 | 2.0±0.1 | 5.1±0.3 | 24.3±3.1 |
| | SF-vent. | 8.0±.4 | 3.5±0.2 | 71.7±0.3 | 1.4±0.04 | 5.4±0 | 10.9±1 | 7.4±0.1 | 26.9±5.0 |
| | FF-vent. | 8.5±.7 | 6.7±0.3 | 170.1±5.1 | 1.9±0.3 | 6.7±.3 | 20.1±2 | 12±1.5 | 41.9±7.3 |
| **ACC (2.5µM)** | | | | | | | | | |
| | Sealed | 4.7±.3 | 0.5±0.03 | 9.7±0.3 | 0.3±0.03 | 1.9±.1 | 0.7±0.1 | 2.9±0.1 | 7.1±0.5 |
| | Diff. vent. | 7.1±.5 | 0.7±0.03 | 43.1±1.7 | 0.50±.08 | 3.1±.1 | 1.3±0.5 | 3.1±0.7 | 17.1±1.9 |
| | SF-vent. | 7.7±.4 | 3.6±0.3 | 69.7±0.3 | 1.4±0.02 | 5.2±0 | 9.9±0.1 | 3.4±0.1 | 26.9±5.0 |
| | FF-vent. | 8.1±.3 | 6.6±0.9 | 167.1±3.7 | 1.7±0.5 | 6.1±.7 | 18.9±3 | 10.9±2 | 34.7±2.6 |
| **$AgNO_3$ (2.5µM)** | | | | | | | | | |
| | Sealed | 5.4±.4 | 0.7±0.5 | 12.2±0.3 | 0.4±0.03 | 2.3±0.1 | 1.4±0.1 | 3.3±0.1 | 17.9±5.1 |
| | diff. vent. | 5.5±0.2 | 0.8±0.2 | 13.5±0.6 | 0.4±0.01 | 2.3±0.1 | 1.4±0.2 | 3.4±0.2 | 28.9±4.9 |
| | SF-vent. | 5.7±0.4 | 0.7±0.3 | 14.7±0.3 | 0.4±0.00 | 2.3±0.1 | 1.5±0.1 | 3.4±0.1 | 36.9±5.0 |
| | FF-vent. | 6.0±.3 | 1.1±0.2 | 15.9±0.3 | 0.4±0.02 | 2.4±.1 | 1.6±0.1 | 3.5±0.1 | 47.2±3.1 |

*10 days old seedlings; each measurement is for the best 2 (out of 4) seedlings from each of 10 tubes and represents a mean ± SE of 20 seedlings; SF-vent = slow flow ventilation (flow rate = 1.5 $cm^3$ $min^{-1}$); FF-vent = fast flow ventilation (flow rate = 3.5 $cm^3$ $min^{-1}$); Diff. vent = capped with a polypropylene disc. Seedlings grown at *ca.* 25°C in continuous light ( PAR = 150 µmol $m^{-2}s^{-1}$).

†mean maximum root length (3 roots from each seedling ).

# PLATE : 5.01

*In vitro* tobacco seedlings, after 10 days, grown in 60 ml glass vessels containing half strength MS medium (10 ml) lacking any hormone, and under different treatments:

(A) ACC at 2.5 μM concentration was added in the medium; each vessel was fitted with a fast flow (FF) convective flow unit (flow rate = 3.5 $cm^3$ $min^{-1}$); note leaves are large green and healthy (X2).

(B) ACC at 2.5 μM concentration was added in the medium; each vessel was fitted with a slow flow (SF) convective flow unit (flow rate = 1.0 $cm^3$ $min^{-1}$); note leaves are quite large but starting to become yellow (X2).

(C) ACC at 2.5 μM concentration was added in the medium; each vessel was capped with a polypropylene disc; note leaves are quite small and yellow (X2).

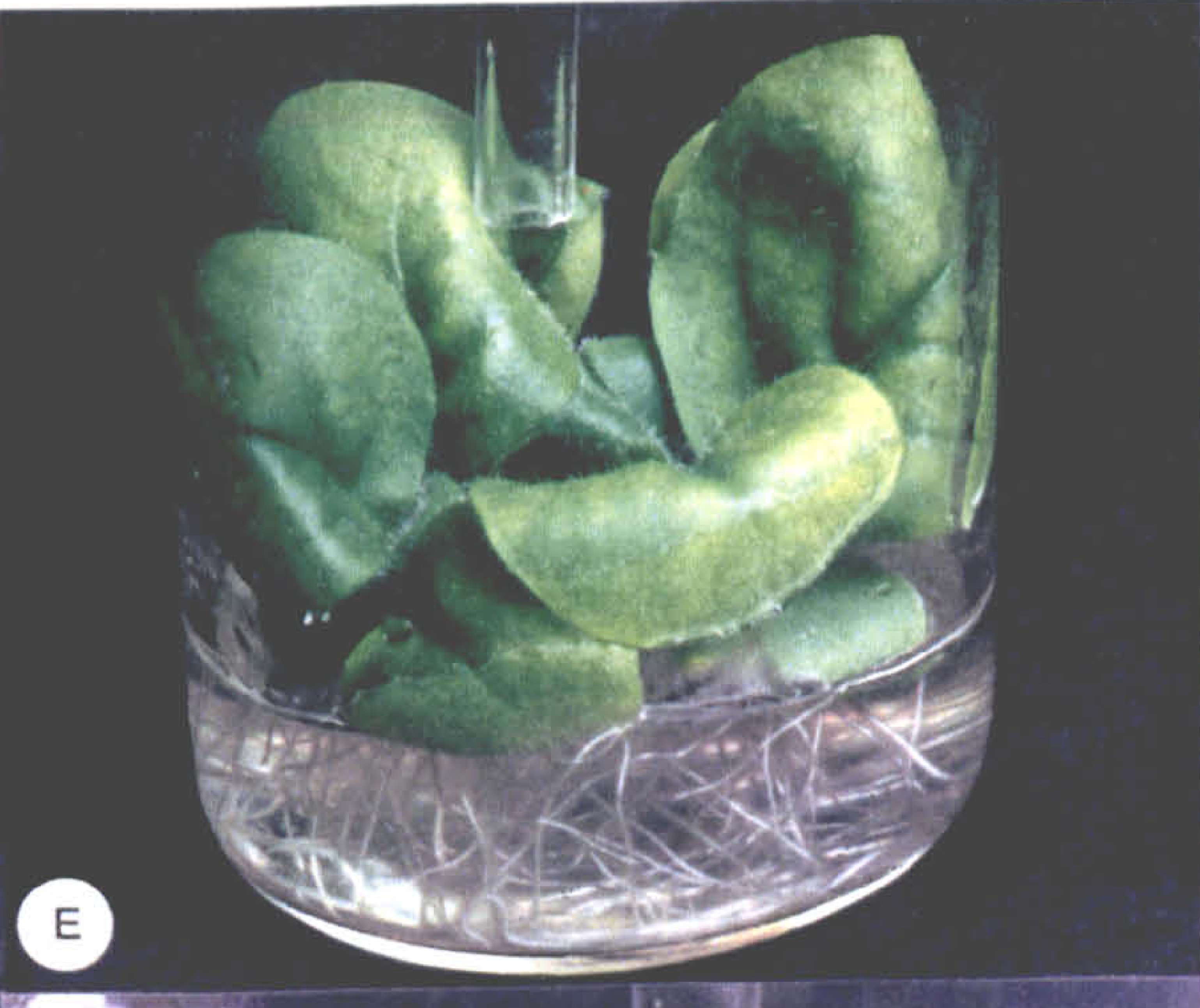
(D) ACC at 2.5 μM concentration was added in the medium; each vessel was sealed with a silicone rubber bung; note leaves are very small and somewhat distorted with epinastic curvature of leaves; also roots sometime grew out of the medium (X2).

(E) control without additives to the medium; each vessel was fitted with a fast flow (FF) convective flow unit (flow rate = 3.5 $cm^3$ $min^{-1}$); note leaves are large green and healthy (X2).

(F) control without additives to the medium; each vessel was fitted with a slow flow (SF) convective flow unit (flow rate = 1.0 $cm^3$ $min^{-1}$); note leaves are quite large but starting to become yellow (X1.5).

(G) control without additives to the medium; each vessel was capped with a polypropylene disc; note leaves are quite small and yellow but larger than those in the ACC treatment (C) (X2).

(H) control without additives to the medium; each vessel was sealed with a silicone rubber bung; note leaves are very small and somewhat distorted with epinastic curvature of leaves; also roots sometime grew out of the medium (X2).

A

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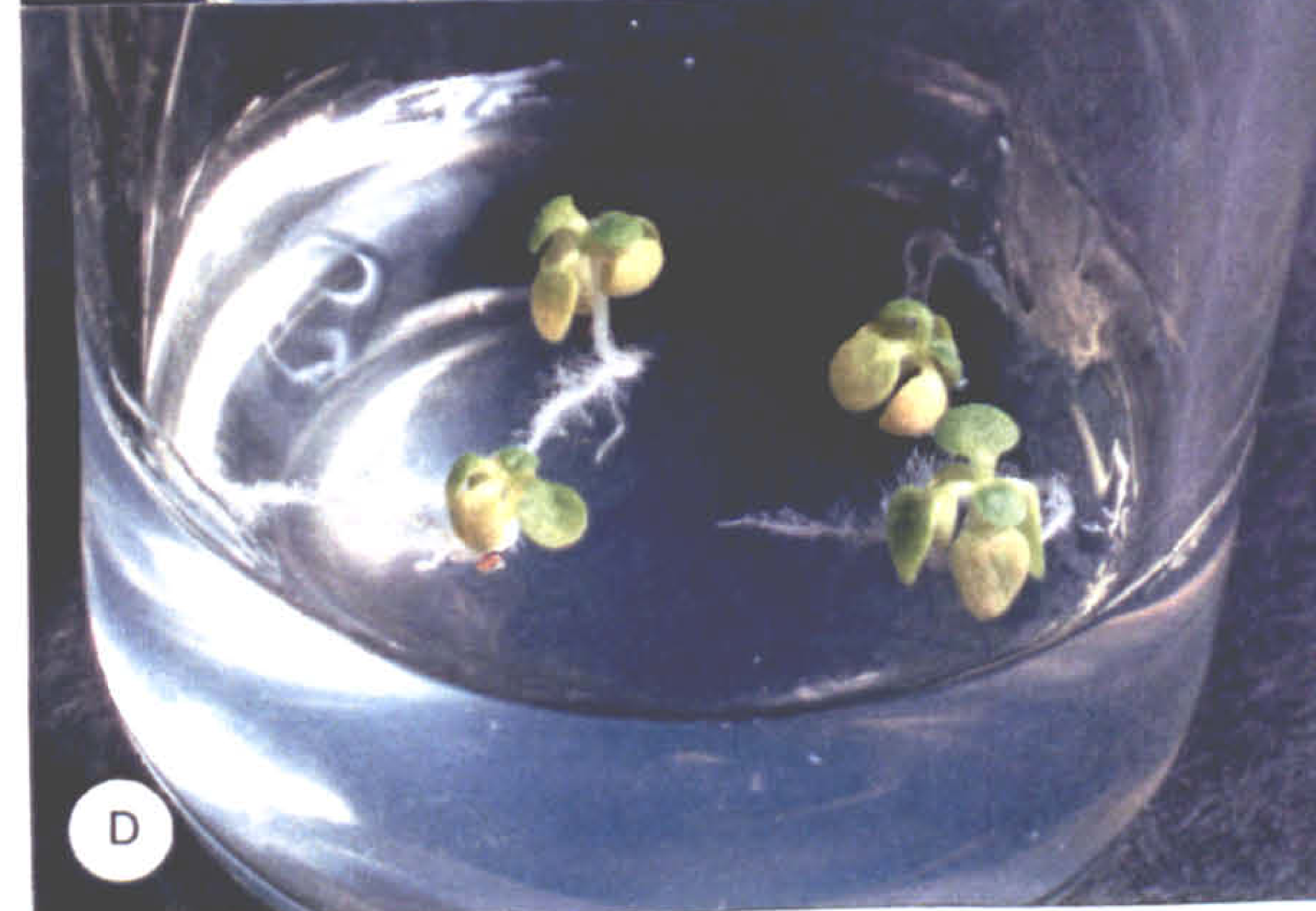
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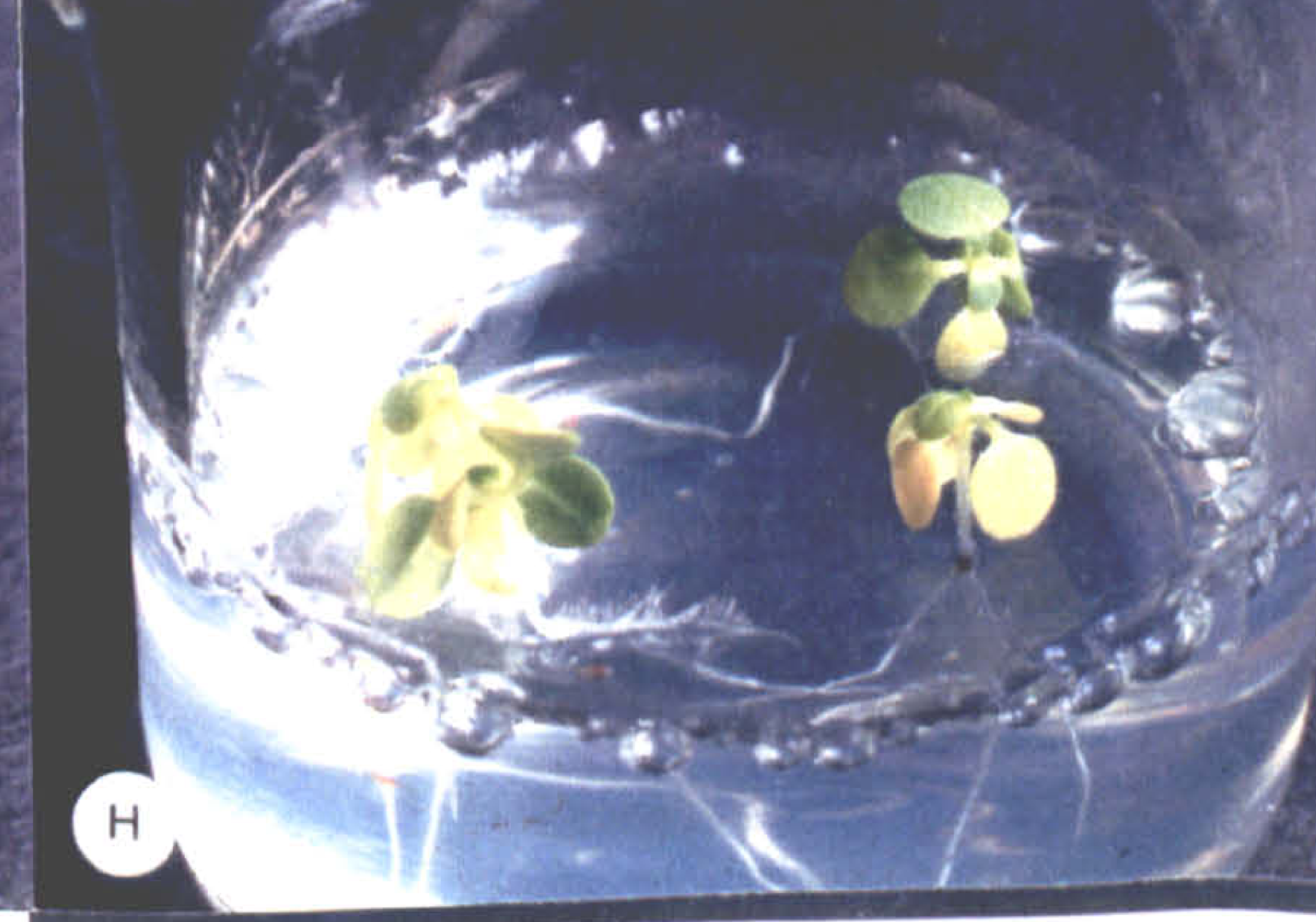

H

lengths were almost double that with SF-ventilation, and although leaf numbers were not greatly different, leaf fresh weight with FF-ventilation was more than double that with SF-ventilation.

#### 5.3.2.2. Growth (with $AgNO_3$)

With silver nitrate at a concentration of 2.5 μM, growth was little different from that of the sealed controls without additives (Table 5.02). However, in all the ventilated treatments growth was substantially inhibited compared with their additive-free controls. For example, comparing FF-ventilation treatments, leaf fresh weights, stem fresh weights and root numbers were respectively <0.1X, <0.1X and <0.3X those of the additive-free controls.

In contrast with the additive-free controls, improved ventilation only marginally improved the growth, the only comparatively large effect was on maximum root lengths.

The poor growth in these treatments would appear to have been due to a toxicity effect of the silver nitrate at this concentration.

#### 5.3.2.3. Growth (with ACC)

Growth in the sealed and diffusively-ventilated ACC treatments (Table 5.02; Plate 5.01) was less than in the additive-free controls; the leaves were yellow and showed epinastic curvature especially under sealed conditions; also the roots sometimes grew out of the medium.

Growth inhibition was particularly evident with the diffusive ventilation treatments, and this suggests that there may have been some ethylene-inhibition of growth resulting from the presence of ACC. With convective flow ventilation, however, growth improved in a similar fashion to that observed with the additive-free controls, and this could be explained in terms of a flushing out of ethylene from the system. This accords with ethylene levels detected in the various treatments (see Fig. 5 04).

Comparing all the growth parameters of the seedlings (Table 5.02) it can be concluded that the fast flow ventilation system showed the best performance. It was also revealed that the growth of tobacco seedlings were completely retarded in sealed vessels probably because of $CO_2$ deficiency in the head-space of the culture vessels although some contribution from accumulated ethylene in both the sealed and diffusive ventilation systems cannot be ruled out. For the diffusive ventilation system with ACC there was a marked lowering of leaf fresh weight compared with the additive-free control.

Thus the enrichment of growth in both the diffusive and forced ventilation may have been a result of both enhanced $CO_2$ concentration (achieved by diffusion and ventilation respectively) and the removal of accumulated ethylene from the culture vessels by the ventilation apparatus.

**5.3.2.4. Photosynthesis (with and without ACC or $AgNO_3$)**

Seedlings subjected to diffusive or forced ventilation (both fast and slow) exhibited significantly higher photosynthetic rates than those grown in sealed vessels (Table 5.03). The photosynthetic rates presented in the table are those achieved (i) at atmospheric $CO_2$ levels (i.e. net atmospheric photosynthetic rate - APR) and (ii) at the $CO_2$ levels created by the plants growing in that particular ventilating system (net working *in vitro* photosynthetic rate - IPR); each has been expressed in terms of mol $plant^{-1}s^{-1}$ and mol $m^{-2}$ leaf surface $s^{-1}$. Carbon dioxide scavenging rates for the additive-free controls are shown in Fig. 5.06.

In sealed conditions without additives, the $CO_2$ scavenging activities of the plants had created an atmosphere with persistently low $CO_2$ levels (*e.g.* 42 $\mu l\ l^{-1}$) and this corresponded with very poor growth (Table 5.02) presumably since it resulted in the plants permanently experiencing a $CO_2$ supply at or close to the compensation point. Consequently the potential rate of photosynthesis (APR) and the actual rates (IPR) were very low (Table 5.03).

**Table 5.03. Effects of different types of ventilation on photosynthetic rates of tobacco seedlings.**

| Treat-ments | Ventilation | *Net photosynthetic atmospheric rate (APR) | | †Net working photosynthetic *in vitro* rate -IPR | |
|---|---|---|---|---|---|
| | | pmol plant$^{-1}$ s$^{-1}$ | μmol m$^{-2}$ s$^{-1}$ | pmol plant$^{-1}$ s$^{-1}$ | μmol m$^{-2}$ s$^{-1}$ |
| **Control** | Sealed | 39 ± 10 | 0.78 ± 0.01 | ≤ 0 | ≤ 0 |
| | Diffusive ventilation | 600 ± 90 | 8.6 ± 0.1 | 110 ± 46 | 1.59 ± 0.03 |
| | Forced ventilation (SF) | 910 ± 20 | 5.2 ± 0.2 | 348 ± 20 | 1.99 ± 0.03 |
| | Forced ventilation (FF) | 2560 ± 35 | 10.9 ± 0.3 | 985 ± 70 | 4.96 ± 0.03 |
| **ACC (2.5 μM)** | Sealed | 30 ± 10 | 0.69 ± 0.1 | ≤ 0 | ≤ 0 |
| | Diffusive ventilation | 530 ± 12 | 7.9 ± 0.14 | 104 ± 80 | 1.45 ± 0.04 |
| | Forced ventilation (SF) | 810 ± 20 | 8.0 ± 0.2 | 309 ± 20 | 1.85 ± 0.05 |
| | Forced ventilation (FF) | 2580 ± 60 | 10.3 ± 0.09 | 836 ± 74 | 3.34 ± 0.02 |
| **$AgNO_3$ (2.5 μM)** | Sealed | 56 ± 10 | 0.89 ± 0.09 | ≤ 0 | ≤ 0 |
| | Diffusive ventilation | 279 ± 50 | 5.57 ± 0.04 | 95 ± 20 | 0.91 ± 0.02 |
| | Forced ventilation (SF) | 360 ± 20 | 5.98 ± 0.09 | 188 ± 20 | 0.93 ± 0.01 |
| | Forced ventilation (FF) | 293 ± 15 | 5.85 ± 0.01 | 294 ± 0.09 | 1.08 ± 0.01 |

*Photosynthetic rates measured at 350 μl l$^{-1}$ $CO_2$ and 72 μmol m$^{-2}$ s$^{-1}$ light flux. †Photosynthetic rates measured at known $CO_2$ concentrations of the culture vessels during the experimental period. Each value represents a mean ± SE of 5 replicates.

❖SF - slow flow ventilation (flow rate = 1.0 - 1.5 cm$^3$ min$^{-1}$); FF - fast flow ventilation (flow rate = 3.5 cm$^3$ min$^{-1}$); for sealed and diffusive ventilation vessels were capped with silicone rubber bungs and polypropylene discs respectively; volume of culture vessel = 60 cm$^3$. Seedlings grown at *ca.* 25°C in continuous light; PAR = 150 μmol m$^{-2}$s$^{-1}$.

Also, in the additive-free treatments, increasing ventilation (diffusive → SF → FF) was accompanied by corresponding increases in $CO_2$ levels (Table 5.04), photosynthesis (Table 5.03) and growth (Table 5.02): photosynthesis reached the highest levels with FF ventilation. For example, with FF, the APR and IPR were remarkably 14X and 29X respectively those in the sealed vessels. Furthermore, with the forced ventilation the $CO_2$ levels were relatively high despite the larger size and hence greater $CO_2$ scavenging activities of the plants. This result is a particularly good illustration of the beneficial effects of forced ventilation. Illustrated differently: with FF-ventilation the IPR in the additive free treatment is 0.45X the rate at atmospheric levels of $CO_2$, with SF ventilation this figure is 0.38X, with diffusive ventilation it falls to 0.18X;. in the sealed treatment it is of course zero.

Similar effects were evident in the ACC treatments except in the diffusive and SF systems where potential photosynthesis was somewhat lower than in the additive-free controls. Even so in the FF treatment, APR and IPR were 15X and 24X higher than in the sealed system. In the case of the diffusive system it can be seen that higher ethylene levels had accompanied the ACC addition (see Fig. 5.04), and this might account for the result. In the case of the SF system no ethylene was detectable in the gas-space, but this does not rule out some slight endogenous inhibitory effect.

As expected, the poorly developed plants in the 2.5 μM silver nitrate treatment exhibited comparatively low rates of photosynthesis per plant and this probably accounts for the higher $CO_2$ levels in the diffusive and forced ventilation compared with additive-free controls. The $CO_2$ concentrations in the sealed vessels with silver were marginally greater than in the additive-free controls, although it is not clear what might have been the reason for this. On the other hand photosynthesis *per* plant was greater where there was diffusive or forced ventilation since the plants were bigger and presumably benefiting from a more favourable $CO_2$ supply.

These results are in agreement with the findings of Fujiwara, Kozai and Watanabe (1987), Fujiwara, Kozai and Watanabe (1988), Kozai and Iwanami (1988),

Kozai, Koyama and Watanabe (1988) and Solárová *et al.* (1989) who postulated that the elevation of $CO_2$ inside culture vessels should increase net photosynthesis.

### 5.3.2.5. Chlorophyll and carotenoid contents

The lowest chlorophyll content, based on fresh weight of leaves, was observed in the sealed vessels in all of the treatments (control, ACC and $AgNO_3$) (Fig. 5.04). The addition of silver at 2.5 μM in sealed conditions may have slightly increased the chlorophyll a content but the difference was barely significant. However, the addition of ACC resulted in a lower chlorophyll content under diffusive ventilation compared with the additive-free control. With forced ventilation, but particularly FF, chlorophyll contents were highest: in both additive-free controls and the ACC treatments chlorophyll contents with FF ventilation were >6.5X those in the sealed vessels. The difference in chlorophyll contents between slow flow and diffusive flow ventilation was greater in the ACC treatment where the diffusive flow system had significant ethylene accumulation (see Fig. 5.05) but none could be detected in the SF ventilation treatment because of the flushing effect of the throughflow convection. Indeed the presence of ACC in the medium had no effect on chlorophyll content with either FF or SF ventilation. In both additive-free and ACC treatments, FF ventilation resulted in chlorophyll contents which were >1.5X those in the SF ventilation. Conversely it is apparent that 2.5 μM silver had a markedly depressing effect on chlorophyll contents in SF, FF and diffusive ventilation treatments; one reason could be the toxicity effect of silver at this concentration.

Similar relationships were observed for carotenoid contents except that in the additive-free and ACC treatments carotenoid levels were almost equally increased by both types of forced ventilation. In the presence of 2.5 μM silver the carotenoid concentrations were severely depressed in all treatments.

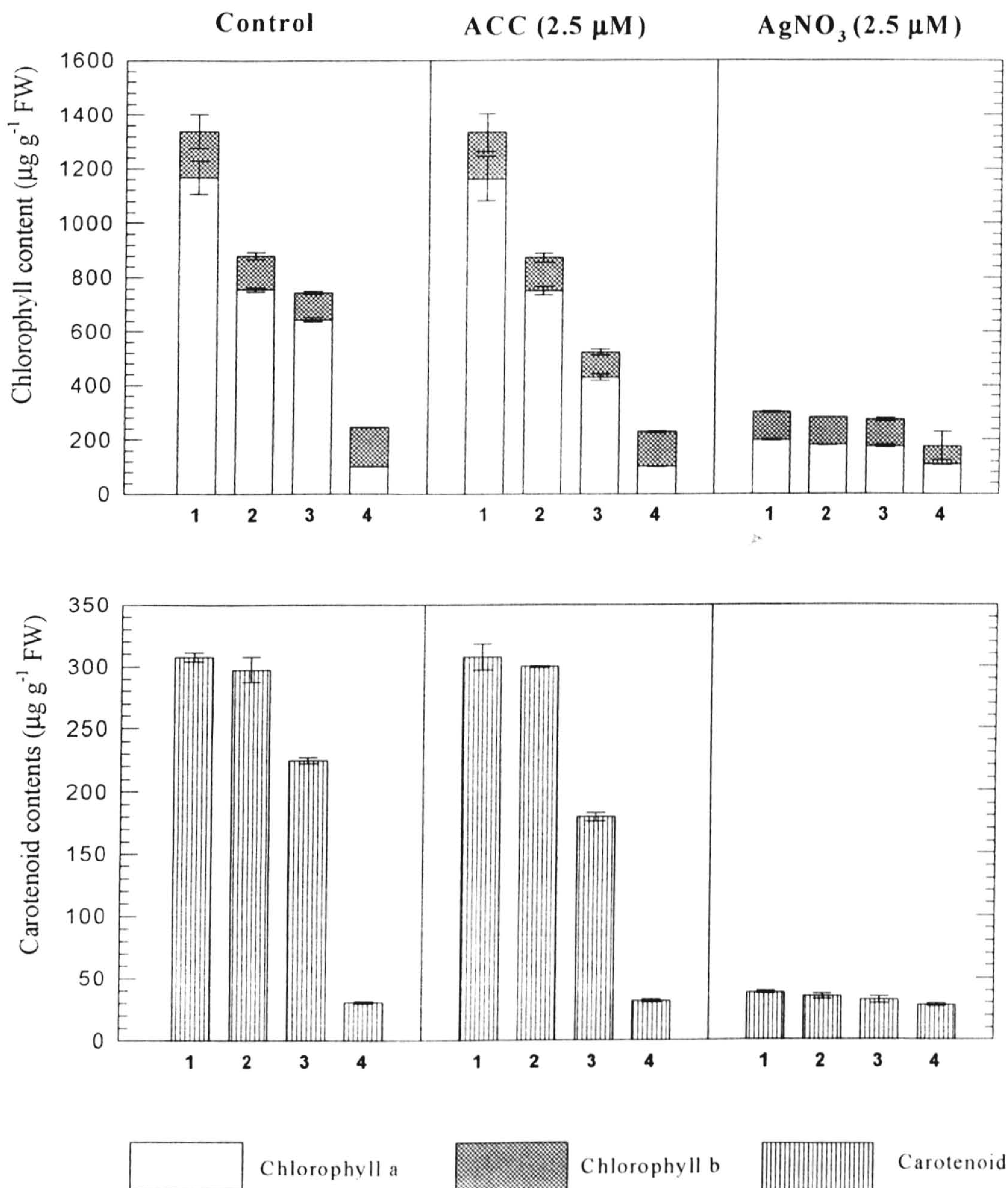



**Fig. 5.04.** Effects of closed, diffusive and forced ventilation systems and presence and absence of ethylene precursor (2.5 μM ACC) and inhibitor (2.5 μM $AgNO_3$) in the rooting medium (MS - ½ strength) on chlorophyll and carotenoid contents of the leaves of *in vitro*-grown 10 days old tobacco seedlings; (1 = FF-ventilation apparatus, 2 = SF-ventilation apparatus, 3 = diffusive ventilation - capped with polypropylene disc and 4 = sealed with silicone rubber bung; each bar represents a mean ± SE of 5 replicates; SF - slow flow ventilation (flow rate=1.0-1.5 $cm^3$ $min^{-1}$); FF - fast flow ventilation (flow rate=3.5 $cm^3$ $min^{-1}$).

### 5.3.3. Effects of different methods of capping on gaseous atmosphere in the culture vessels

#### 5.3.3.1. Ethylene

Highest concentrations of ethylene were found in the sealed vessels in presence of ACC in the nutrient medium (Fig. 5 05). The concentration, 0.41 $\mu l\ l^{-1}$, was >2X that of the additive-free sealed control and silver treatments. Compared to the sealed vessels those with diffusive ventilation contained even lower concentrations of ethylene: 0.07 $\mu l\ l^{-1}$ in the additive-free control, 0.12 $\mu l\ l^{-1}$ with ACC, and 0.058 $\mu l\ l^{-1}$ in the presence of 2.5 $\mu M$ silver.

Wherever the SF or FF ventilation apparatus was employed, virtually no ethylene was found in the head-space of the culture vessels.

The results seem to demonstrate a significant impedance to ethylene loss with the diffusive flow ventilation and an efficient flushing with both types of forced ventilation.

#### 5.3.3.2. Carbon dioxide

During the light period the atmosphere of the head-space of sealed vessels in all the treatments (ACC, $AgNO_3$ and control) contained significantly lower concentrations of $CO_2$ when compared with those having diffusive and forced ventilation (Table 5.04). This was almost certainly due to the photosynthetic activity of the seedlings; since the only additional source of $CO_2$ would be respiratory in origin, severe $CO_2$ depletion must result in such a system. The equilibrium concentration was about 42 $\mu l\ l^{-1}$ $CO_2$ in the additive-free control and ACC treatments, and a little higher in the sealed plus silver treatment. These figures represent the $CO_2$ compensation values (Fig. 5.06) and the results strongly correlate with the findings presented in Table 5.02 which demonstrated that growth was very poor in sealed vessels.

With the vessels capped with polypropylene membrane to provide diffusive ventilation, $CO_2$ concentrations increased significantly in the additive-free and ACC treatments. The concentrations increased with increasing efficiency of ventilation, the

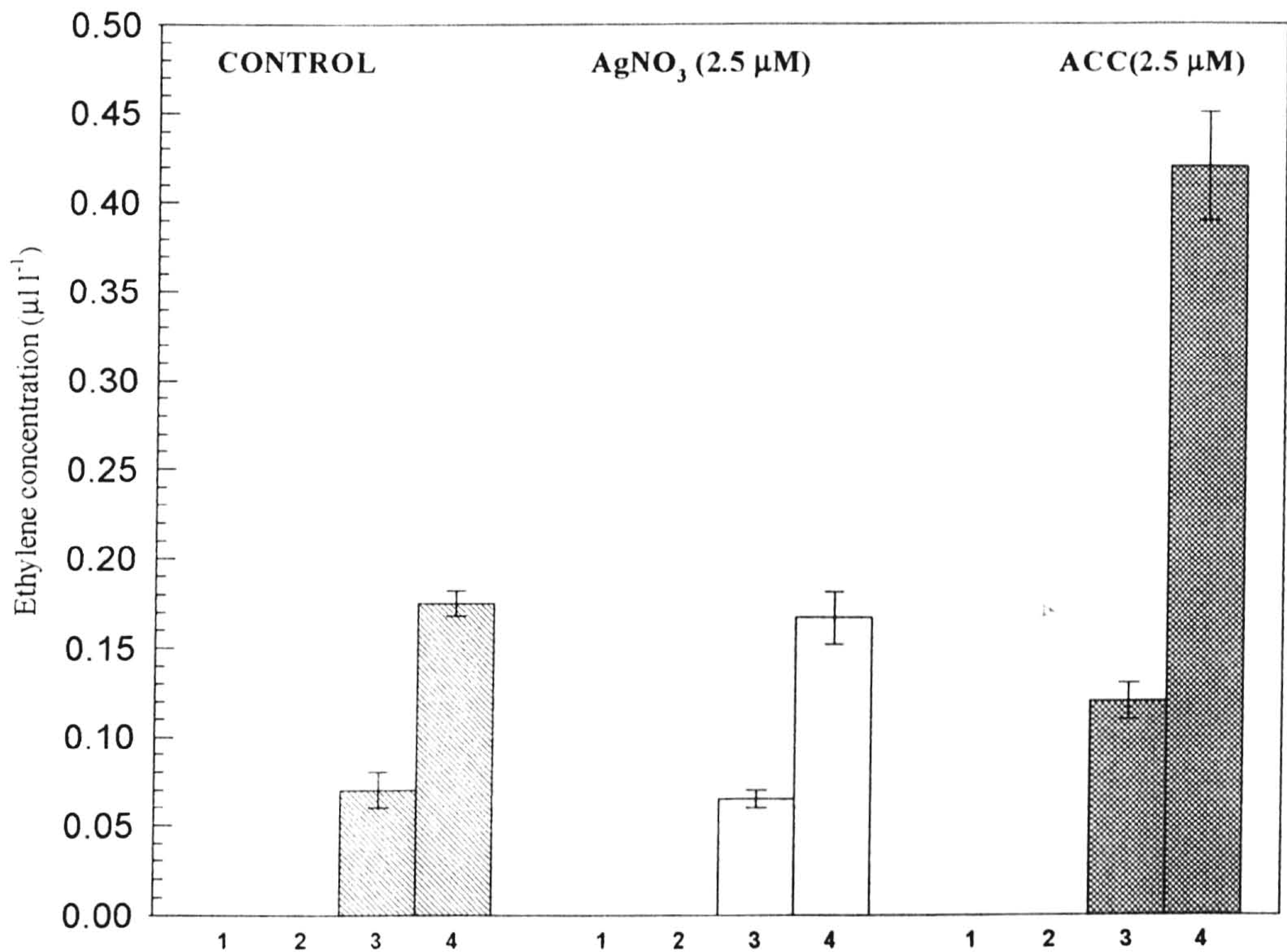



**Fig 5.05.** Effects of closed, diffusive and forced ventilation and presence and absence of ACC (2.5 μM) and $AgNO_3$ (2.5 μM) in the rooting medium (MS - ½ strength) on the ethylene concentrations in the culture head-space; tobacco seedlings were 10 days old; ① = fast flow ventilation (flow rate = 3.5 $cm^3$ $min^{-1}$); ② = slow flow ventilation (flow rate=1.0-1.5 $cm^3$ $min^{-1}$); ③ = diffusive ventilation capped with polypropylene disc; ④ = sealed with silicone rubber bung; each bar represents a mean ± SE of 5 - 7 replicates. Seedlings were grown at *ca.* 25°C in continuous light; PAR = 150 μ mol $m^{-2}$ $s^{-1}$.

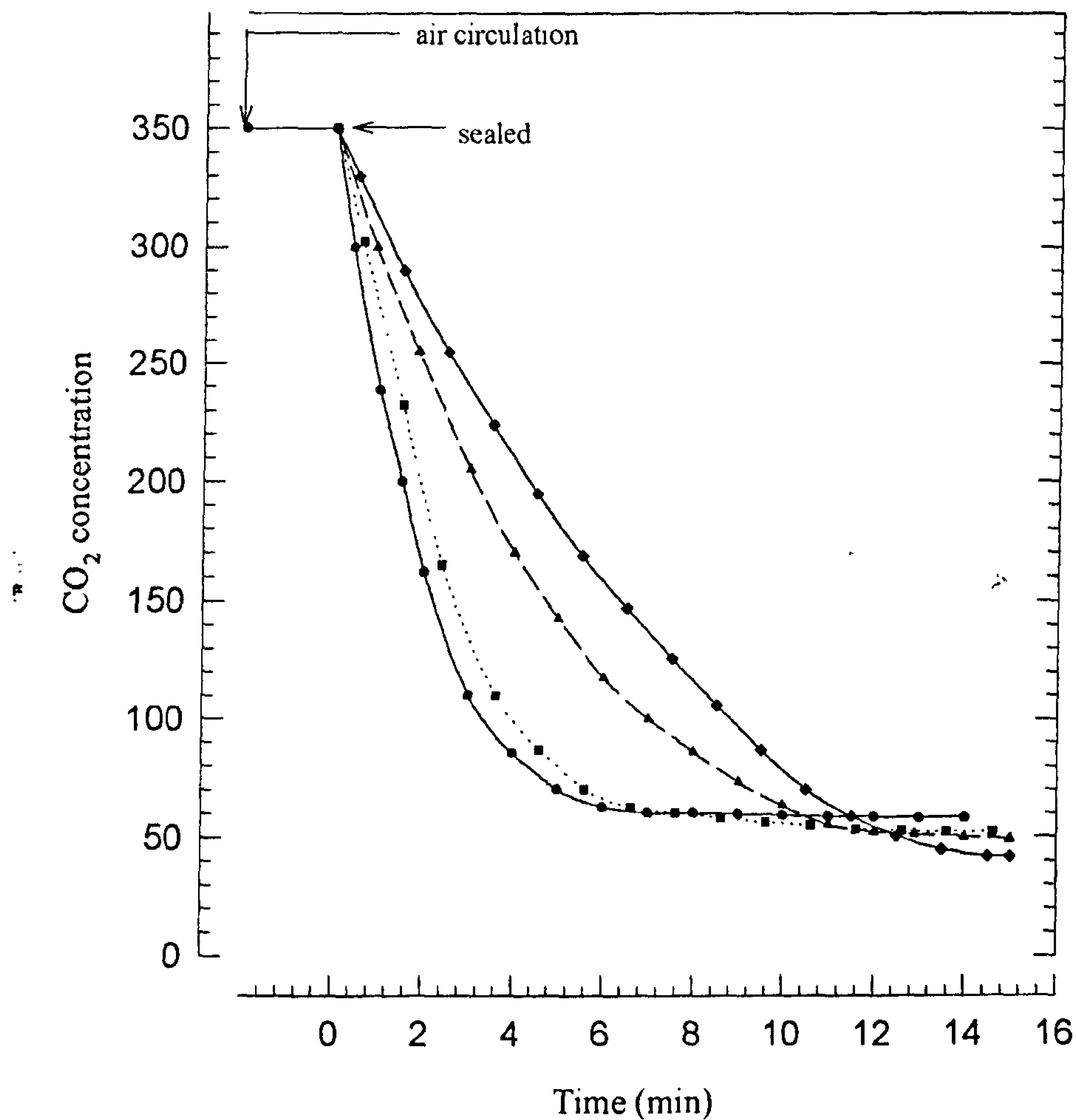



**Fig. 5.06.** Showing the depletion of $CO_2$ in the head-space of culture vessels containing 10-day-old tobacco seedlings (at 72 μmol $m^{-2}$ $s^{-1}$ PAR); seedlings had been previously grown under fast flow ventilation (flow rate=3.5 $cm^3$ $min^{-1}$) (●), slow flow ventilation (flow rate=1.0 - 1.5 $cm^3$ $min^{-1}$) (■), diffusive ventilation (capped with polypropylene disc) (▲), and under sealed condition (with silicone rubber bung) (◆).

highest being reached with the FF ventilation (>3.5X those in the sealed treatments) and concomitantly, higher rates of photosynthesis (Table 5.03) and better growth (Table 5.02) occurred. Again it should be noted that despite the better growth and higher photosynthetic rates the FF treatment was able to sustain higher $CO_2$ levels than the diffusive or SF systems. However, an even faster flow would be required to fully satisfy the scavenging capability of the plant (see Table 5.03).

**Table 5.04. Effects of different types of ventilation, ACC and $AgNO_3$ on carbon dioxide concentrations ($\mu l\ l^{-1}$) in the culture vessel atmosphere of tobacco seedlings (10 days old) exposed to continuous light (150 $\mu mol\ m^{-2}\ s^{-1}$ PAR).**

| Treatments | Sealed with silicone rubber bung | Diffusive ventilation (capped with polypropylene disc) | Forced ventilation (Slow flow; rate = 1.0 $cm^3\ min^{-1}$) | Forced ventilation (Fast flow; rate = 3.5 $cm^3\ min^{-1}$) |
|---|---|---|---|---|
| Control | 41.7 ± 7.0 | 62.3 ± 11.3 | 108.3 ± 7.5 | 146.0 ± 12.1 |
| ACC (2.5 μM) | 42.1 ± 5.4 | 64.5 ± 10.1 | 110.1 ± 7.2 | 149.8 ± 12.5 |
| $AgNO_3$ (2.5 μM) | 59.8 ± 6.6 | 143.8 ± 13.1 | 235.8 ± 19 | 334.5 ± 15 |

* Each value represents a mean ± SE of 7 - 10 replicates.

With the addition of 2.5 μM $AgNO_3$, $CO_2$ levels again increased with increasing efficiency of ventilation. However, the actual levels were much higher than above and these can be explained in terms of lower rates of photosynthesis, lower chlorophyll content and poorer growth of the plants due to silver toxicity.

From these results it seems reasonable to suppose that although there is some evidence that ethylene can have a suppressing effect on growth e.g. in the diffusive flow system, it is the elevation of $CO_2$ concentrations produced by the ventilation apparatus which was probably mainly responsible for the increased growth and development of tobacco seedlings. To explore further the effects of $CO_2$ enhancement, another

experiment was conducted by applying higher concentrations of $CO_2$ in the atmosphere of the culture vessels (see Section 5.3.4).

### 5.3.4. Growth and development of seedlings with diffusive ventilation: elevation of $CO_2$ and exogenous ethylene

It should first be noted that in this experiment although the ventilation was diffusive, the system may not be directly comparable with the previous examples in this chapter since the external atmosphere was confined within a large sealed glass chamber. Consequently, there was more chance of boundary layer effects developing above the polypropylene membrane and a greater possibility of endogenous ethylene accumulation in the culture vessels. Indeed ethylene levels in the culture vessels in this experiment varied between 0.1 and 0.4 $\mu l\ l^{-1}$ with 350 $\mu l\ l^{-1}$ $CO_2$ in the chamber compared with only 0.07 $\mu l\ l^{-1}$ when the vessels were in the open growth room (Section 5.3.3.1). It is not known what levels were achieved at the higher $CO_2$ concentrations.

#### 5.3.4.1. Growth

The best performance was observed in the 3000 $\mu l\ l^{-1}$ $CO_2$ treatment (Figs. 5.07 and 5.08; Plate 5.02). Here the fresh weight of leaves was 6.4X and the leaf area 3.6X those of the controls where the seedlings were subjected to atmospheric levels of $CO_2$: 350 $\mu l\ l^{-1}$. The number of leaves at 3000 $\mu l\ l^{-1}$ $CO_2$ was also higher: 1.3X than that of the 350 $\mu l\ l^{-1}$ $CO_2$ control. Also, the root systems were remarkably better with 22 roots per plant equivalent to 3X that of the control; similarly, stem fresh weights and lengths were increased: respectively 8X and 1.9X those of the controls. At 1500 $\mu l\ l^{-1}$ $CO_2$, growth was generally intermediate between the 350 and 3000 $\mu l\ l^{-1}$ $CO_2$ treatments except for maximum root lengths which were similar to those in the 3000 $\mu l\ l^{-1}$ treatment. At 0 - 5 $\mu l\ l^{-1}$ $CO_2$, growth was very poor but was appreciably increased at 350 $\mu l\ l^{-1}$ $CO_2$.

It is interesting to note that the growth attained under normal additive-free FF-ventilation (see Table 5.02) lies approx. between that of the 1500 and 3000 $\mu l\ l^{-1}$ $CO_2$ treatments. Thus, throughflow ventilation can compensate for a lack of $CO_2$ enrichment.

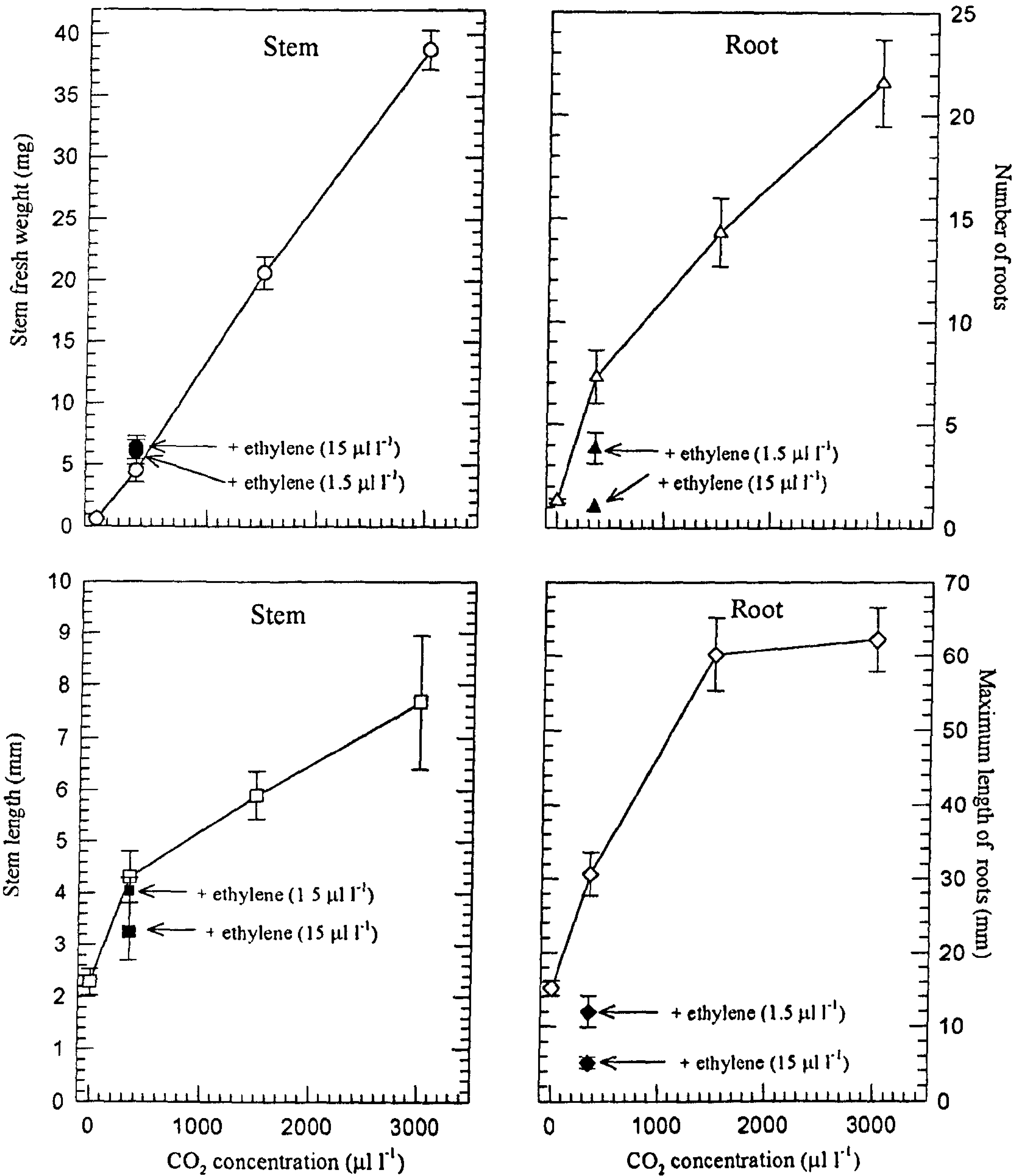



**Fig. 5.07.** Effects of elevation of $CO_2$ and exogenous ethylene in the culture head-space on stem and root growth of 15 days old, *in vitro*-grown tobacco seedlings; 60 ml culture vessels were each capped with a polypropylene disc and enclosed in a 5000 ml glass chamber; each chamber was recharged as intervals (Section 5.2.2.3.) with appropriate gas concentrations; each symbol represents a mean ± SE of 10 replicates.

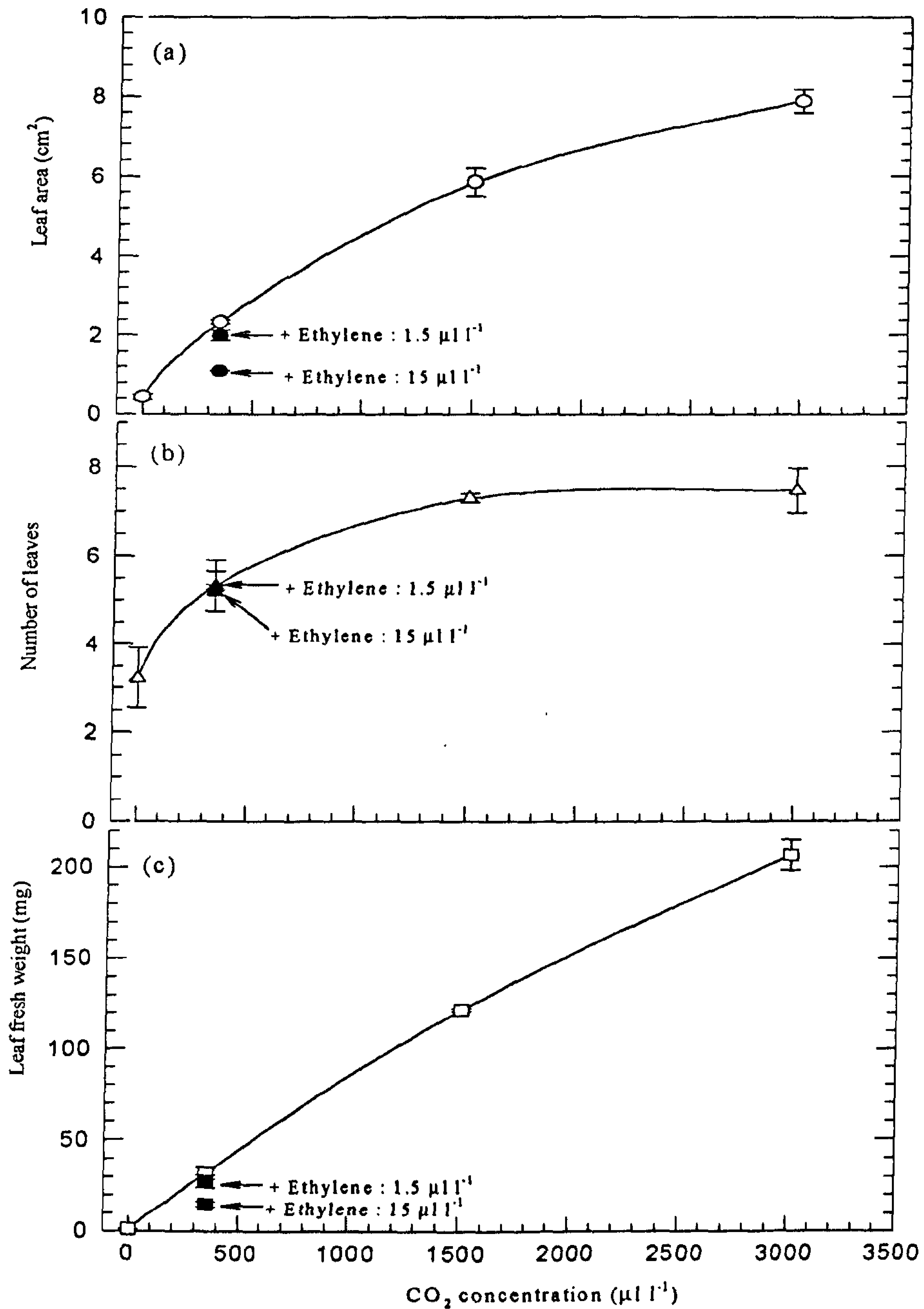



**Fig. 5.08.** Effects of elevation of $CO_2$ and exogenous ethylene in the culture head-space on (a) leaf area (b) leaf number and (c) leaf fresh weight of 15 days old *in vitro* grown tobacco seedlings; 60 ml culture vessels were each capped with a polypropylene disc and enclosed in a 5000 ml glass chamber; each chamber was recharged at intervals (Section 5.2.2.3.) with appropriate gas concentrations; each symbol represents a mean ± SE of 10 replicates.

Also, however, it may be seen that the vessel $CO_2$ levels in the 350 μl $l^{-1}$ $CO_2$ treatment are similar to those of the SF-ventilation in the earlier experiments. This possibly indicates some ethylene-inhibition of growth in the present experiment.

When exogenous ethylene (15 μl $l^{-1}$) was added with the 350 μl $l^{-1}$ $CO_2$ concentration, then all the growth factors apart from stem fresh weight and number of leaves were appreciably reduced in comparison to the controls (350 μl $l^{-1}$ $CO_2$ with no exogenous ethylene). Also one effect of the ethylene seemed to be to make the petioles elongate (Plate 5.02). The most significant growth retardation was found in the root systems where numbers of roots were 0.12X and the maximum lengths were 0.17X those of the controls. At a concentration of 1.5 μl $l^{-1}$ ethylene, which is much closer to values naturally attained in sealed cultures, it can be seen that there was again some growth inhibition; the effect was greatest on root numbers and lengths, and there was a small but significant reduction in leaf area. These results clearly show that high dosages of ethylene can have very deleterious effects on the growth and development of tobacco seedlings, but also that the concentrations likely to occur in sealed containers can cause some growth retardation. Huxter, Thorpe and Reid, (1981) and Bolton and Freebairn (1975) have also reported on the deleterious effects of high ethylene dosage.

From these results it can be concluded that there is a very strong positive correlation between $CO_2$ concentration in the culture vessels and the growth of *in vitro* grown tobacco seedlings. Also, however, there are again strong indications that the small levels of ethylene accumulating in diffusive ventilation treatments may be sufficient to depress growth significantly. Again, the data strongly support the need for the forced ventilation of culture vessels.

### 5.3.4.2. Effective carbon dioxide concentration

The $CO_2$ concentration in the culture vessels and the chamber were measured every 3 days during the experimental period. As shown in Fig. 5.09, except for the 0 - 5 μl $l^{-1}$ treatment, the $CO_2$ concentrations measured in the culture vessels atmospheres became

## PLATE : 5.02

*In vitro* tobacco seedlings, after 15 days, grown in 60 ml glass vessels containing half strength MS medium (10 ml) lacking any hormone, and under different $CO_2$ and ethylene concentrations in the culture head-space:

(A) 0 - 5 $\mu l\ l^{-1}$ $CO_2$ concentration; note extremely stunted seedlings and yellow leaves (X2.7).

(B) 350 $\mu l\ l^{-1}$ $CO_2$ concentration; seedlings stunted but larger than in (A) and still the leaves are yellow (X2.7).

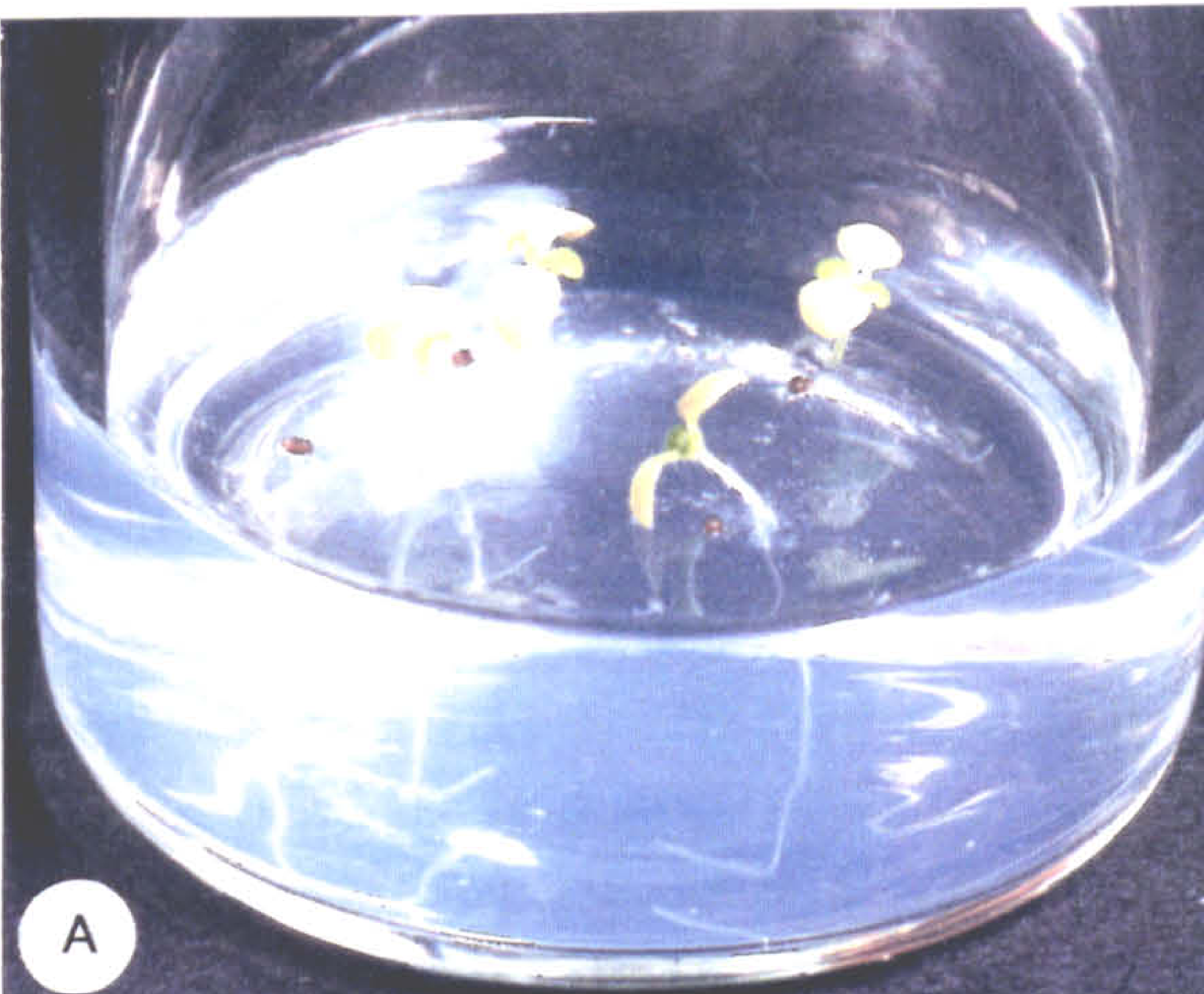
(C) 350 $\mu l\ l^{-1}$ $CO_2$ concentration + 1.5 $\mu l\ l^{-1}$ ethylene; note seedlings are very stunted but slightly larger than (D); leaves are yellow with more elongated petioles than in (A) (X2.7).

(D) 350 $\mu l\ l^{-1}$ $CO_2$ concentration + 15 $\mu l\ l^{-1}$ ethylene; note seedlings are very stunted, leaves are yellow with more elongated petioles than in (A) (X2.7).

(E) 1500 $\mu l\ l^{-1}$ $CO_2$ concentration; note good growth; leaves remained green (X2.7).

(F) 3000 $\mu l\ l^{-1}$ $CO_2$ concentration; note good growth; leaves remained green (X2.7).

*Culture vessels were each capped with a polypropylene disc and enclosed in 5000 ml glass chamber; each chamber was recharged at intervals (Section 5.2.2.3.) with appropriate gas concentrations; each symbol represents a mean ± SE of 10 replicates.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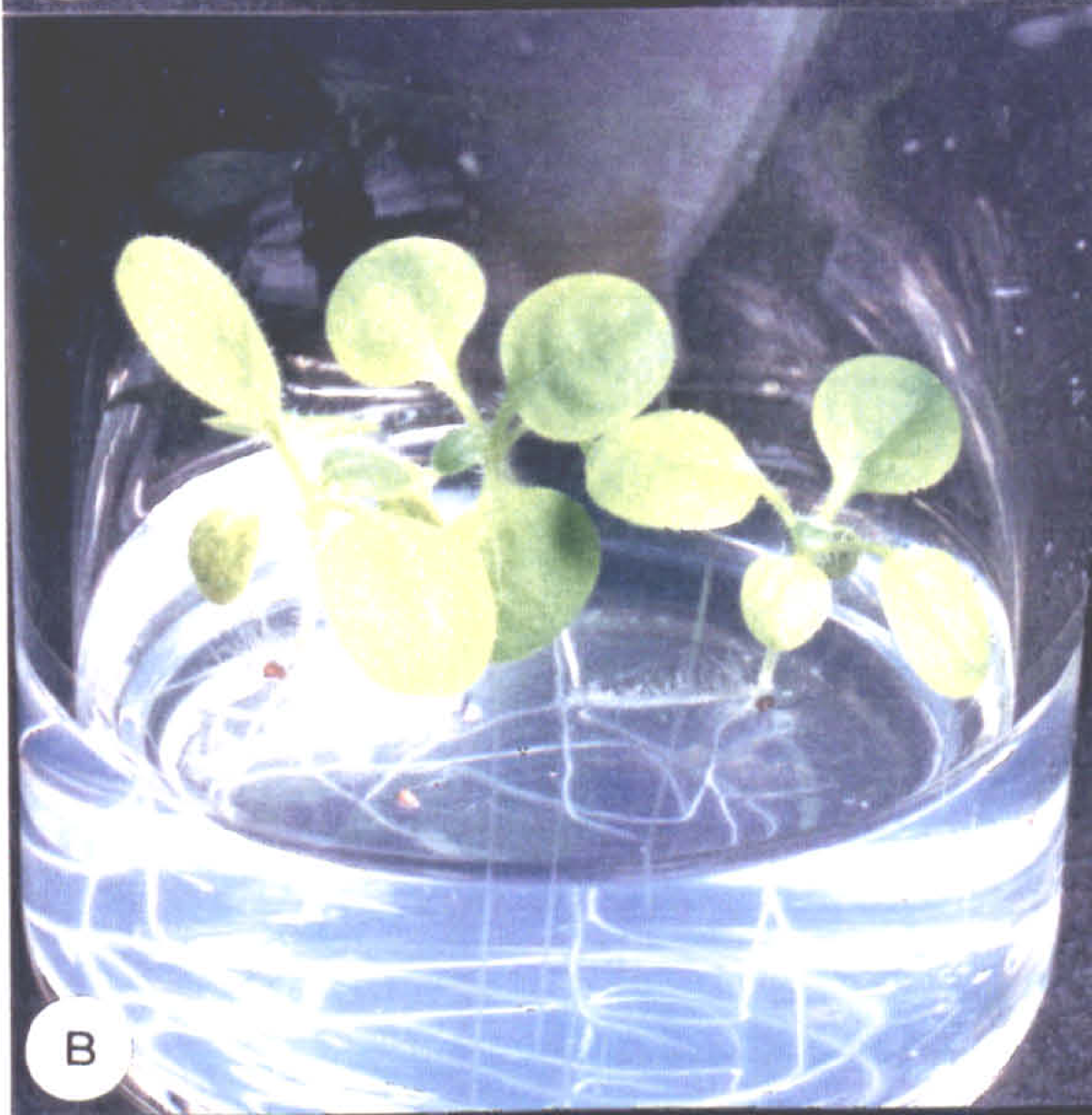
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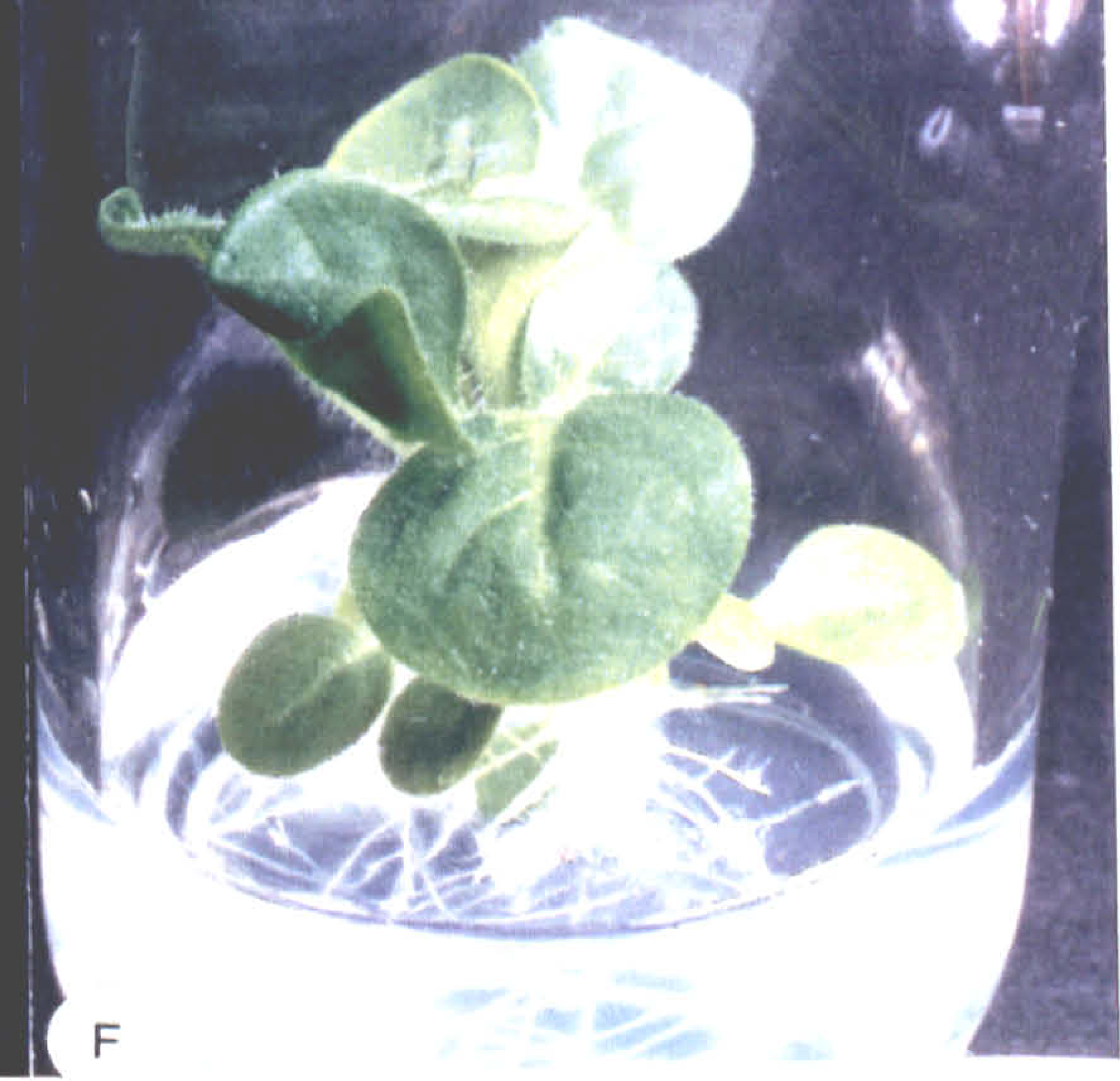
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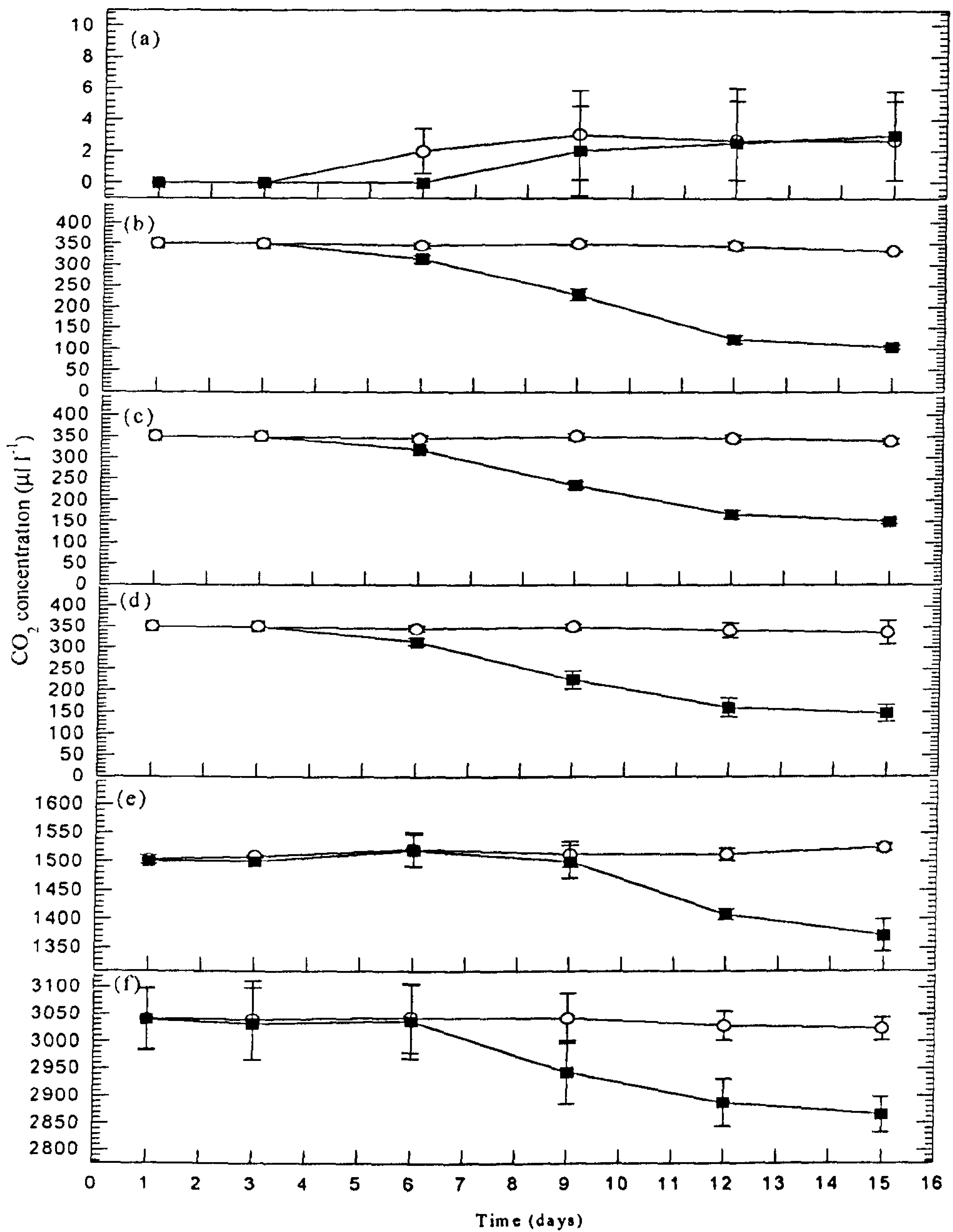



**Fig. 5.09.** $CO_2$ concentration in the enclosing chamber head-space (O) and in the culture vessels' head-space (■) containing *in vitro*-grown tobacco seedlings; 60 ml vessels were capped with polypropylene disc and enclosed in a *5000 ml* glass chamber; each chamber was recharged at intervals (Section 5.2.2.3) with (a) 0 - 5 µl $l^{-1}$ $CO_2$ (b) 350 µl $l^{-1}$ $CO_2$ (c) 350 µl $l^{-1}$ $CO_2$ + 1.5 µl $l^{-1}$ ethylene (d) 350 µl $l^{-1}$ $CO_2$ + 15 µ$l^{-1}$ ethylene (e) 1500 µl $l^{-1}$ $CO_2$ (f) 3000 µl $l^{-1}$ $CO_2$.

significantly lower towards the end of the experiment (8 - 15th day). The reason is undoubtedly due to the increasing $CO_2$ demand of photosynthesis coupled to the membrane diffusive resistance Another important factor which might have influenced the result was transpirational water from the leaf which condensed on the inner side of the polypropylene membrane, which would presumably have increased its resistance. As a consequence diffusion rates through the polypropylene would decrease as would the level of $CO_2$ within the vessel. During the present study it became increasingly apparent that condensation on the polypropylene membranes could be an important drawback to this type of ventilation system.

As described earlier, very small shoot and root systems developed when 0 - 5 µl $l^{-1}$ of $CO_2$ was supplied and thus photosynthetic rate and transpiration rates would also be very low. As a result the inside $CO_2$ concentration was almost similar that of the outer atmosphere (i.e. within the chamber) (Fig 5.09).

**5.3.4.3. Chlorophyll and carotenoid contents**

The chlorophyll contents based on the fresh weight of the leaves of the seedlings increased with increasing $CO_2$ concentrations in the atmosphere of the culture vessels. The greatest difference was between 0 and 350 µl $l^{-1}$ concentrations (Fig 5.10a). At 350 µl $l^{-1}$ $CO_2$ the chlorophyll content was 488 µg $g^{-1}$ fresh weight and this was >40X that at 0-5 µl $l^{-1}$ $CO_2$ concentration. From these results it could be concluded that atmospheric $CO_2$ concentration is directly related to the chlorophyll contents of tobacco leaves.

In the presence of exogenous ethylene in the culture vessels (i e. 15 µl $l^{-1}$ *plus* 350 µl $l^{-1}$ $CO_2$) the chlorophyll content was appreciably reduced to 0.67X that of the control without ethylene. However, even the 1.5 µl $l^{-1}$ $CO_2$ treatment caused a significant decline: the chlorophyll content was 0.76X that of the control. This suggests that ethylene was responsible for the decline in chlorophyll contents of the tobacco leaves. These findings are in agreement with the results of Cournac *et al.* (1991) where the

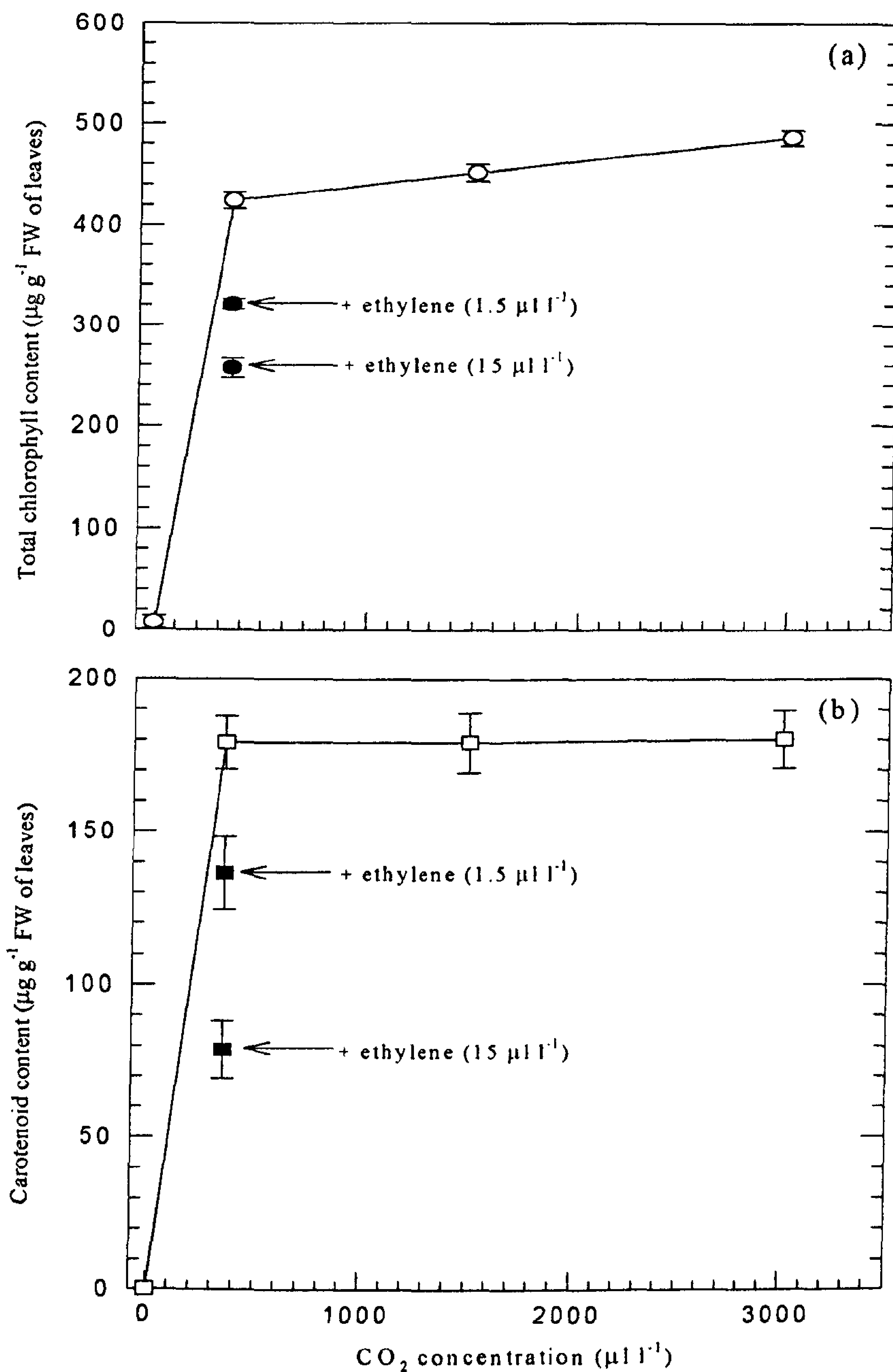



**Fig. 5.10.** Effects of elevated concentrations of $CO_2$ and exogenous ethylene in the culture head-space on (a) total chlorophyll contents and (b) carotenoid contents of leaves of 15 days old *in vitro grown tobacco seedlings; 60 ml culture vessels were capped with* polypropylene disc and enclosed in 5000 ml glass chamber; each chamber was recharged at intervals (Section 5.2.2.3) with appropriate gas concentrations; each symbol represents a mean ± SE of 10 replicates.

chlorophyll contents of *Solanum tuberosum* leaves increased when ethylene inhibitors ($Ag_2S_2O_3$) were added in the medium.

Similar relationships to those for chlorophyll content were found also in the case of the carotenoids.(Fig. 5.10b). They were remarkably low at the lowest $CO_2$ concentration and were more than halved by ethylene in air at 15 μl $l^{-1}$.

**5.3.4.4. Photosynthesis and irradiance**

After 15 days in the various $CO_2$ treatments the relationships between net photosynthesis (μmol $CO_2$ $m^{-2}$ leaf surface $s^{-1}$) and irradiance (PAR, μmol $m^{-2}$ $s^{-1}$) at atmospheric $CO_2$ levels were measured and the results are shown in Fig. 5.11.

The highest rate of photosynthesis (3.88 μmol $m^{-2}$ $s^{-1}$ at 360 μmol $m^{-2}$ $s^{-1}$ PAR) occurred in the plants taken from the 3000 μl $l^{-1}$ $CO_2$ treatment, the rates for the other plants decreased in accordance with the $CO_2$ concentration at which they had been previously grown. The lowest rate (0 23 μmol $m^{-2}$ $s^{-1}$ at 360 μmol $m^{-2}$ $s^{-1}$ irradiance) was for the plants originally grown at 0 - 5 μl $l^{-1}$ $CO_2$. The photosynthetic capacity of these plants was therefore only 0 06X of that of the plants from the 3000 μl $l^{-1}$ $CO_2$ treatment. The results suggest that the photosynthetic potential of tobacco plants can be strongly dependant upon the $CO_2$ concentration in atmosphere in which they have been cultured. However, the net photosynthetic rates found in this experiment were considerably lower than those in the previous one. For example in the 350 μl $l^{-1}$ $CO_2$ treatment the net rate at *ca.* 75 μmol $m^{-2}$ $s^{-1}$ was approx. 0.9 μmol $m^{-2}$ $s^{-1}$ compared with 8.6 μmol $m^{-2}$ $s^{-1}$ in the previous experiment. However, the comparable chlorophyll contents in the current experiment (430 μg $g^{-1}$ F.W.) were only 0.55X that of the diffusive treatment in the previous experiment. Why this should have been so is not clear but, in the present experiment, the culture vessels were enclosed and the ethylene concentrations were greater during the growth period. It seems possible that endogenous ethylene might have been indirectly responsible for the lower rates of photosynthesis. This is supported by the results from the treatments with added ethylene, where photosynthesis declined with

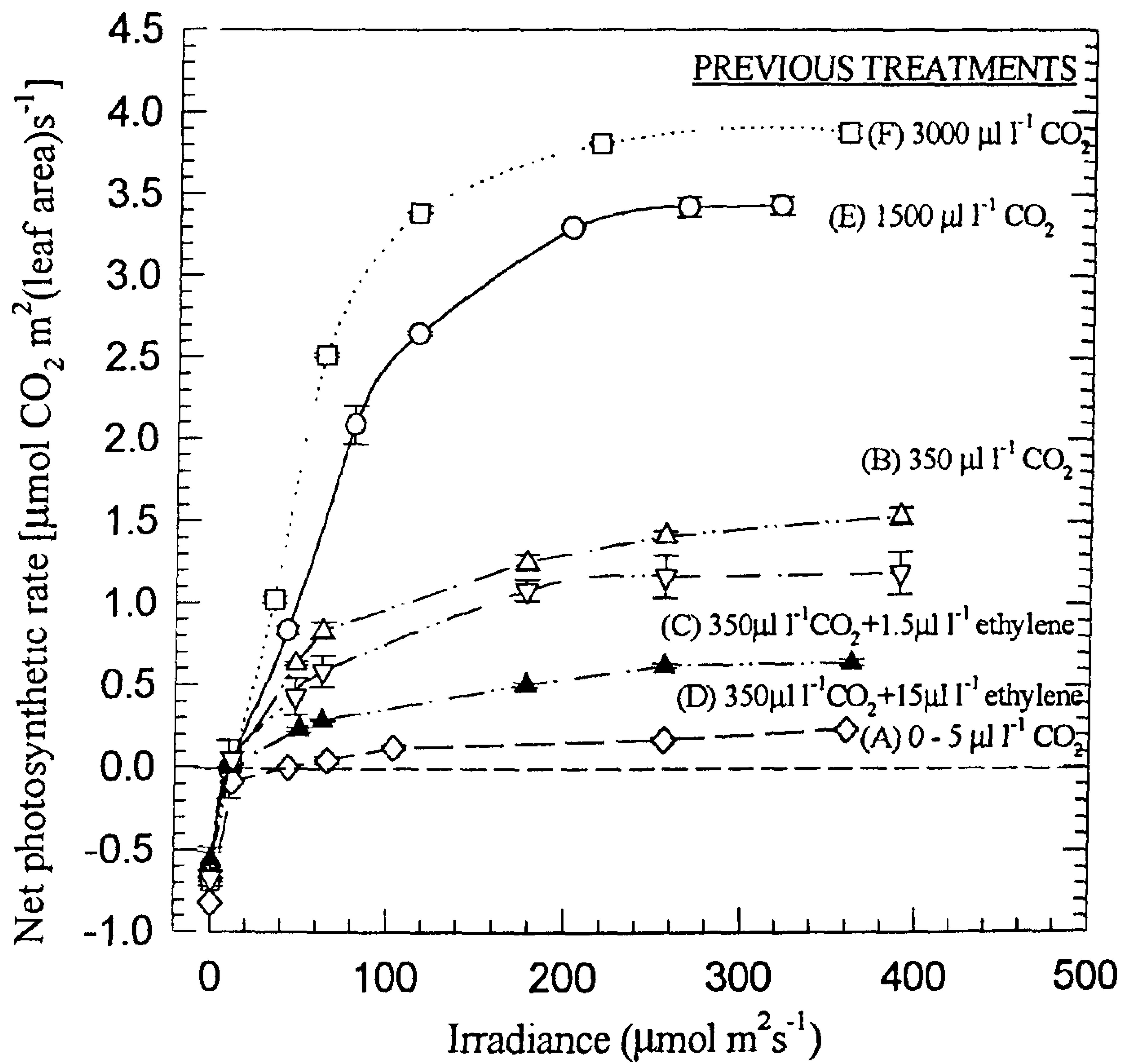



**Fig. 5.11.** The relationship between net photosynthesis and irradiance at atmospheric $CO_2$ levels for tobacco seedlings previously grown for 15 days under the different $CO_2$ and $CO_2$ + ethylene treatments; each symbol represents a mean ± SE of 5 replicates.

increasing ethylene concentrations (Fig. 5.11) and where chlorophyll and carotenoid contents similarly declined (Fig. 5.10). However, it is rather puzzling that although the 1500 and 3000 $\mu l\ l^{-1}$ $CO_2$ treatments more than doubled the potential for photosynthesis, the chlorophyll contents rose by only 1.14X and the carotenoid contents did not change.

To summarise, a strong positive correlation seems to exist between the $CO_2$ concentration in the culture atmosphere and the potential developed for photosynthesis of tobacco seedlings. It can be seen also, that an increased light flux might be beneficial to growth. On the other hand, higher light fluxes can cause greater condensation in the culture vessels, and particularly on polypropylene membranes and hence in the long term might depress growth in diffusively ventilated treatments through impeded ventilation.

### 5.3.5. Shoot culture from cutting: the effects of closed, diffusive and forced ventilation systems

#### 5.3.5.1 Growth and development

Shoot tips inoculated on to culture medium led to new shoot proliferation and callus development. Considering all the growth parameters, the best performance occurred in the cultures subjected to forced ventilation (FF). As shown in Fig. 5.12 and Plate 5.03 the best shoot system was found in forced ventilation with 12.5 mg callus and 297 mg shoot (including leaf) fresh weight; the latter compared with only 120 mg in the sealed system and 125 mg with diffusive ventilation. The number of leaves and maximum length of each shoot also increased remarkably in this treatment and were respectively 2.2X and 4.0X those in the sealed vessels. However, the number of shoots was not significantly higher than that of other two treatments.

Cultures grown under diffusive ventilation exhibited an intermediate growth pattern between that of the forced ventilation and closed system. Therefore, it could be concluded that forced ventilation showed the best performance for improving the growth and development of *in vitro* grown tobacco shoot culture.

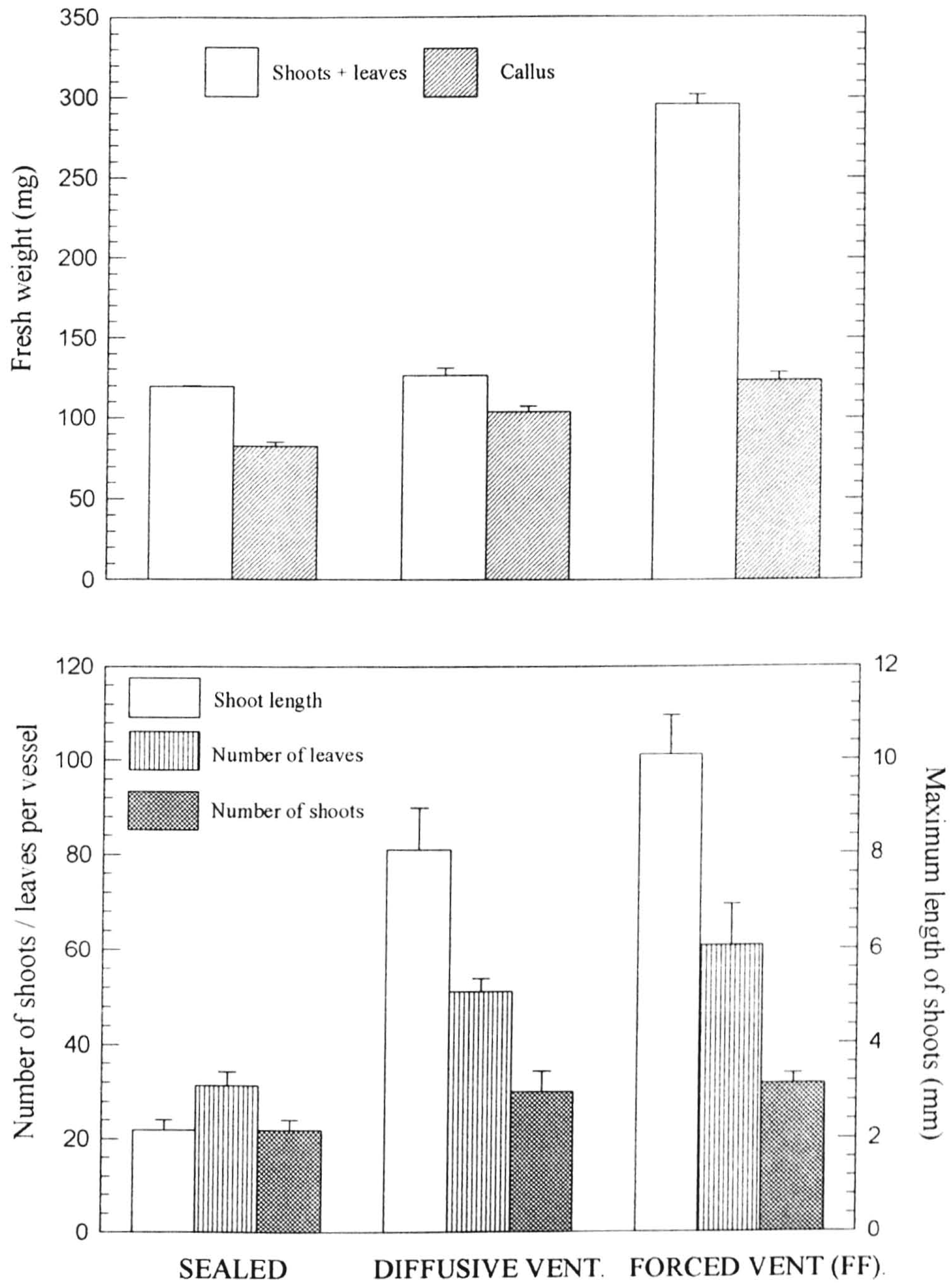



**Fig. 5.12.** Effects of closed, diffusive and forced ventilation (fast flow, flow rate = 3.5 $cm^3$ $min^{-1}$) on growth and development of 30 days old *in vitro*-grown tobacco shoot cultures; each bar represents a mean ± SE of 5-7 replicates.

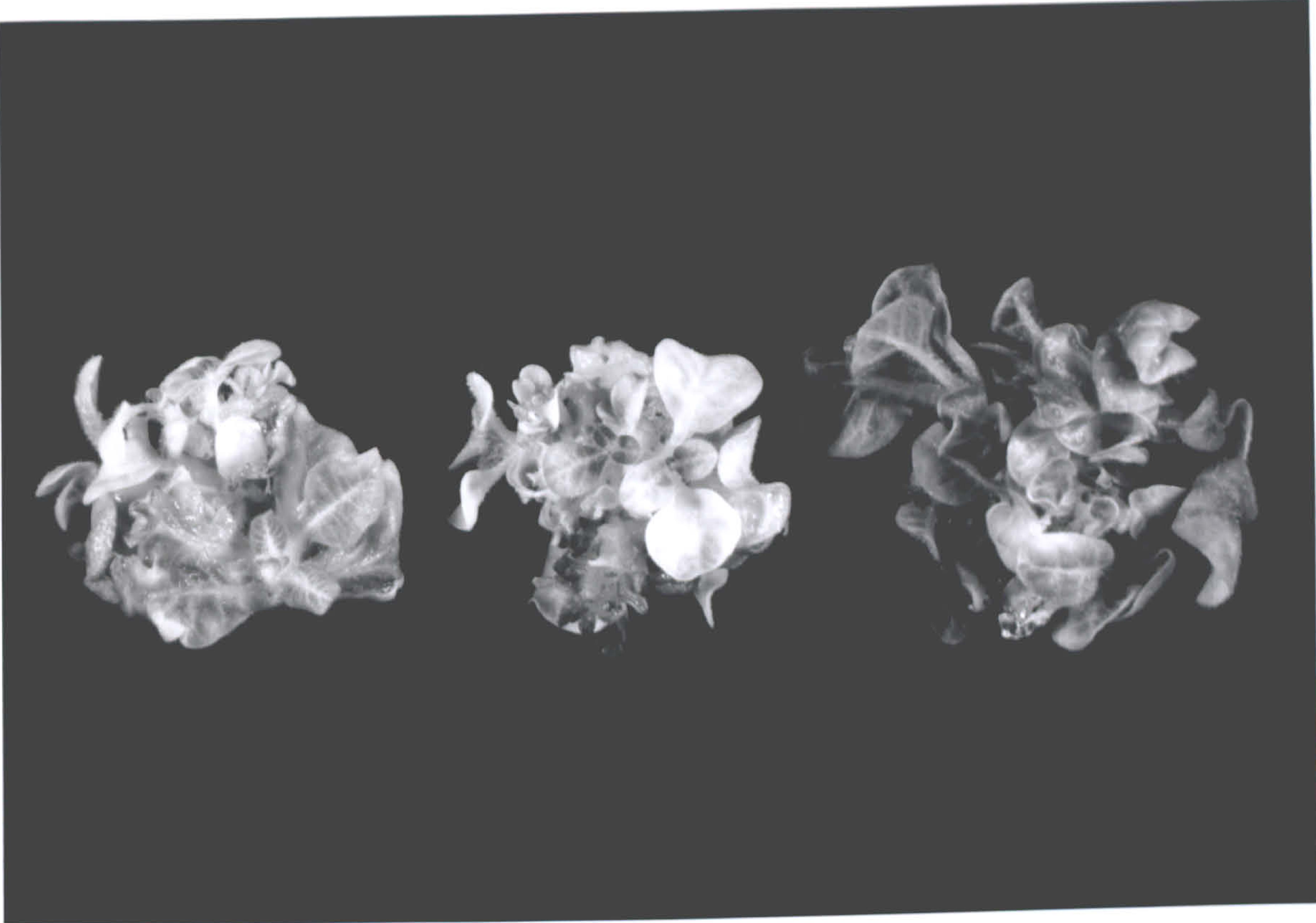
**PLATE : 5.03**

30 days old *in vitro* grown tobacco shoot cultures grown in 60 ml glass vessels containing full strength MS medium (10 ml) supplemented with BAP (0.5 mg $l^{-1}$) + NAA (0.1 mg$l^{-1}$). Vessels were capped as follows:

(Left hand side): silicone rubber bung; note poorer growth and yellowing leaves (1.5X).

(Middle): polypropylene membrane; note poorer growth and yellowing leaves (1.5X).

(Right hand side): fast flow ventilation apparatus (flow = 3.5 $cm^3$ $min^{-1}$); note good healthy growth and green leaves (1.5X).



### 5.3.2.4. Carbon dioxide uptake/production rate

The carbon dioxide uptake/production rates were measured after 30 days of culture. The results showed that the plantlets subjected to forced ventilation were absorbing $CO_2$ at the rate of 12 X $10^{-6}$ $m^3$ $s^{-1}$ $m^{-3}$ (culture) which was 8X that of the diffusive grown cultures. Under sealed conditions, however, there was at this stage no net absorption of $CO_2$, rather they were producing $CO_2$ at the rate of 10.8 X $10^{-6}$ $m^3$ $s^{-1}$ $m^{-3}$ (culture) (Fig. 5.13). This was no doubt due to the small size and the yellow nature of the leaves. These results were consistent with the findings shown in Fig. 5.14 where high amounts of $CO_2$ were noticed in sealed vessels.

### 5.3.2.5. Gaseous atmosphere of the culture vessels

In sealed vessels the ethylene levels (0.1 $\mu l\ l^{-1}$) were 5X greater than those in the diffusive vessels (Fig. 5.14a), while with forced ventilation there was almost no ethylene in the atmosphere of the culture Although the effects of such ethylene accumulation on overall growth of the culture are not clear, the levels are so low that it is tempting to assume that the gas has no deleterious effects, at least not on the callus fresh weight and number of shoots developing (Fig 5.12).

On the other hand, however, the concentration of $CO_2$ was very high in the sealed vessels (2411 $\mu l\ l^{-1}$) (Fig. 5.14b), being respectively 5.6X and 12.4X higher than those of diffusive and forced ventilation systems. (This is a quite different result from that with the tobacco *seedlings* where, in the absence of callus, there was a net absorption of $CO_2$). As shown in Fig. 5.13 the cultures in the closed system were net producers of $CO_2$ rather than net absorbers. In the case of the seedlings, however, 3000 $\mu l\ l^{-1}$ $CO_2$ was associated with better growth and hence this raises the possibility again that even the relatively low level of ethylene in these callus plus shoot cultures might have been sufficient to have depressed growth to some extent. Indeed the poorer growth in the diffusive vessels might have been induced in such a way. It seems reasonable to assume that respiratory activity in the callus of the sealed vessels was masking the

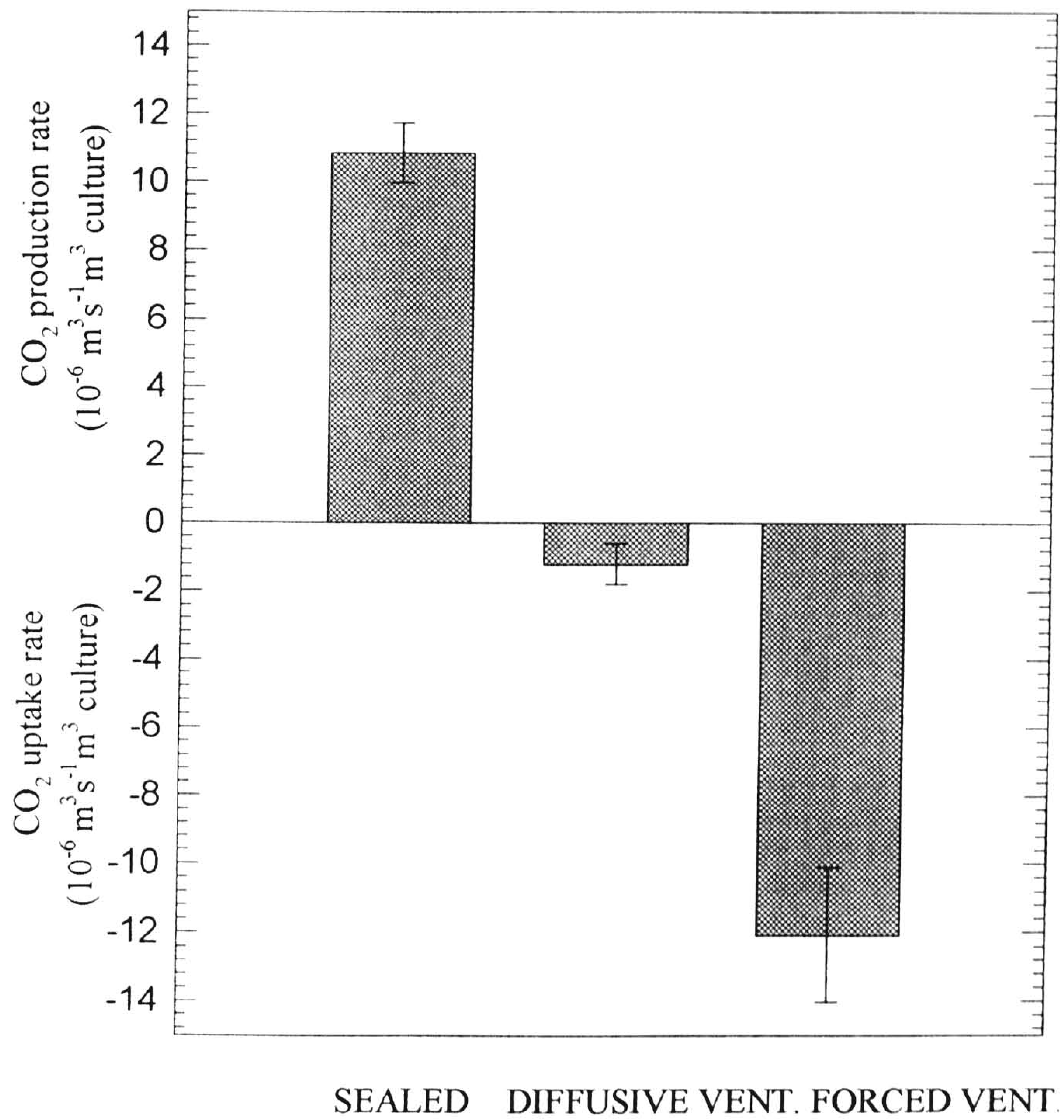



**Fig. 5.13.** Effects of closed, diffusive and forced ventilation (fast flow - flow rate = 3.5 $cm^3$ $min^{-1}$) on $CO_2$ production and rate of uptake of 30 days old *in vitro* grown tobacco shoot cultures; each bar represents a mean ± SE of 5 replicates.

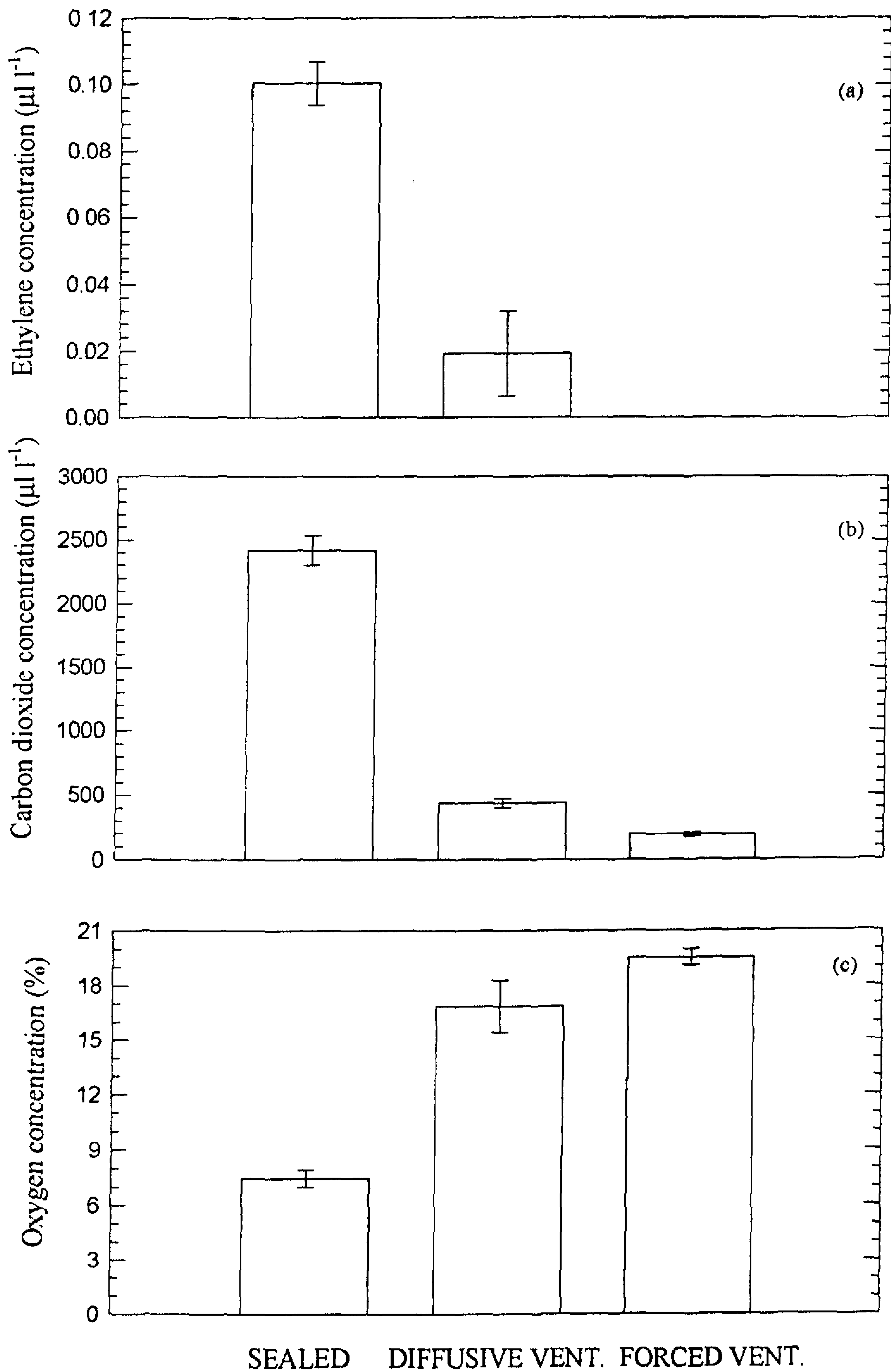



**Fig. 5.14.** Effects of closed, diffusive and forced ventilation (fast flow, flow rate=3.5 $cm^3$ $min^{-1}$) on (a) ethylene (b) carbon dioxide and (c) oxygen concentrations of the culture head-spaces of 30 days old *in vitro*- grown tobacco shoot cultures; each bar represents a mean ± SE of 5-7 replicates.

photosynthetic effect of the green shoots, and in the diffusive vessels the relatively high $CO_2$ levels again suggest that callus respiration was making a very significant contribution Alternatively it may be that tobacco callus produces other volatiles capable of suppressing shoot growth. Unfortunately, with tobacco it was not possible to separate the callus and the shoots; however, the results for cauliflower (Chapter IV: Fig. 4.10) show how important a contribution callus respiration can make to net $CO_2$ exchange.

With forced ventilation the $CO_2$ concentration was noticeably lower (195.2 $\mu l\ l^{-1}$) indicating the more effective scavenging activity of the larger plants.

The concentrations of oxygen were very low in the closed vessels, only 7.5 *per cent* at the end of the experiment (Fig. 5.14c). Although this was unexpected, it is consistent with the dominance of respiration over photosynthesis noted above. With diffusive ventilation the oxygen concentrations were slightly lower (at 17%) than atmospheric, whereas with forced ventilation the value was just below atmospheric. These values no doubt reflected the balance between respiration, photosynthesis and the differences in ventilating efficiency of the two treatments.

### 5.3.5. Growth, chlorophyll contents and yellowing of leaves : the evaluation of the closed system, diffusive and forced ventilation

During the experiments on tobacco seedlings, cuttings or cultures it was interesting to note that the leaves started to become yellow in colour after only a few days of culture in sealed and sometimes in diffusive vessels (Plate 5.04); in the sealed treatment the leaves also showed epinastic curvature. To explore further the responses, an experiment was set up in which the treatments were applied to equally sized plants grown first under forced ventilation. By the end of the experiment it was found that the plantlets subjected subsequently to the closed system or diffusive system had became completely yellowish in colour. The plantlets in slow flow ventilation also showed some signs of yellowing but not to a great extent. It was also established that the fast flow ventilation system was

able to maintain the natural green colour of the leaves in tobacco culture (Plates 5.04; 5.05).

### 5.3.5.1. Growth

Among the treatments the best growth and development were observed in the plantlets subjected to FF ventilation (Figs. 5.15 and 5 16 and Plate 5.05) After only eight days the leaf systems were remarkably better than the plants with closed ventilation (Plate 5.04): the numbers, area and fresh weight of leaves per plant with FF ventilation were approx. 1.4X, 3.3X and 3.0X greater than those of the closed treatments. Similarly root systems were also enhanced significantly in this treatment. The mean maximum length of roots was *ca.*39 mm compared with only 16 mm in the sealed system. Even more strikingly, however, it can be seen that there were few new leaves and no increase in leaf area, and few roots produced neither was there any extension of the longest roots, when plants were transferred from FF to closed ventilation.

Plantlets subjected to diffusive and slow flow ventilation also exhibited better growth (both leaf and root system) than that of sealed grown plantlets. However, as shown in Figs. 5.15 and Fig. 5.16 the overall growth in slow flow ventilation was slightly better than that in the diffusive one.

### 5.3.5.2. Ethylene and carbon dioxide concentration

Before applying the treatments i.e. under forced ventilation, no ethylene accumulation was found in the culture vessel atmosphere. However, as shown in Fig. 5.17a, on the 2nd day of the treatment (9th day of culture) in sealed conditions ethylene concentrations had already become high (almost 0.8 $\mu l\ l^{-1}$) and by the end of the experiment had reached a concentration of 1.28 $\mu l\ l^{-1}$. Under diffusive ventilation the concentration of ethylene also increased but not to such a great extend (0.54 $\mu l\ l^{-1}$). However, in forced ventilation (fast and slow) the head-space of the culture vessels did not accumulate any ethylene.

Almost immediately after applying closed ventilation the $CO_2$ concentrations in these culture vessels became very much lower and at the end of the experiment were *ca.*

## PLATE : 5.04

*In vitro* tobacco cuttings, after 7 days, grown in 60 ml glass vessels containing half strength MS medium (10 ml) lacking any hormone, and under different conditions of ventilation. (The plants had been previously grown 7 days in fast flow ventilation (flow = 3.5 $cm^3$ $min^{-1}$)

upper : vessels were sealed with silicone rubber bungs; note that the leaves became very yellow and distorted with epinastic curvature of leaves (X2).

lower : vessels were capped with polypropylene disc; note that the leaves became yellow (X2).

**PLATE : 5.05**

*In vitro* tobacco cuttings, after 7 days, grown in 60 ml glass vessels containing half strength MS medium (10 ml) lacking any hormone, and under different conditions of ventilation. (The plants had been previously grown 7 days in fast flow ventilation (flow = 3.5 $cm^3$ $min^{-1}$)

upper : each vessel was fitted with a slow flow convective flow unit (flow rate = 1.0 $cm^3$ $min^{-1}$); note leaves becoming only very slightly yellow (X2).

lower: each vessel was fitted with a fast flow convective flow unit (flow rate = 3.5 $cm^3$ $min^{-1}$); note leaves remained healthy and green (X2).

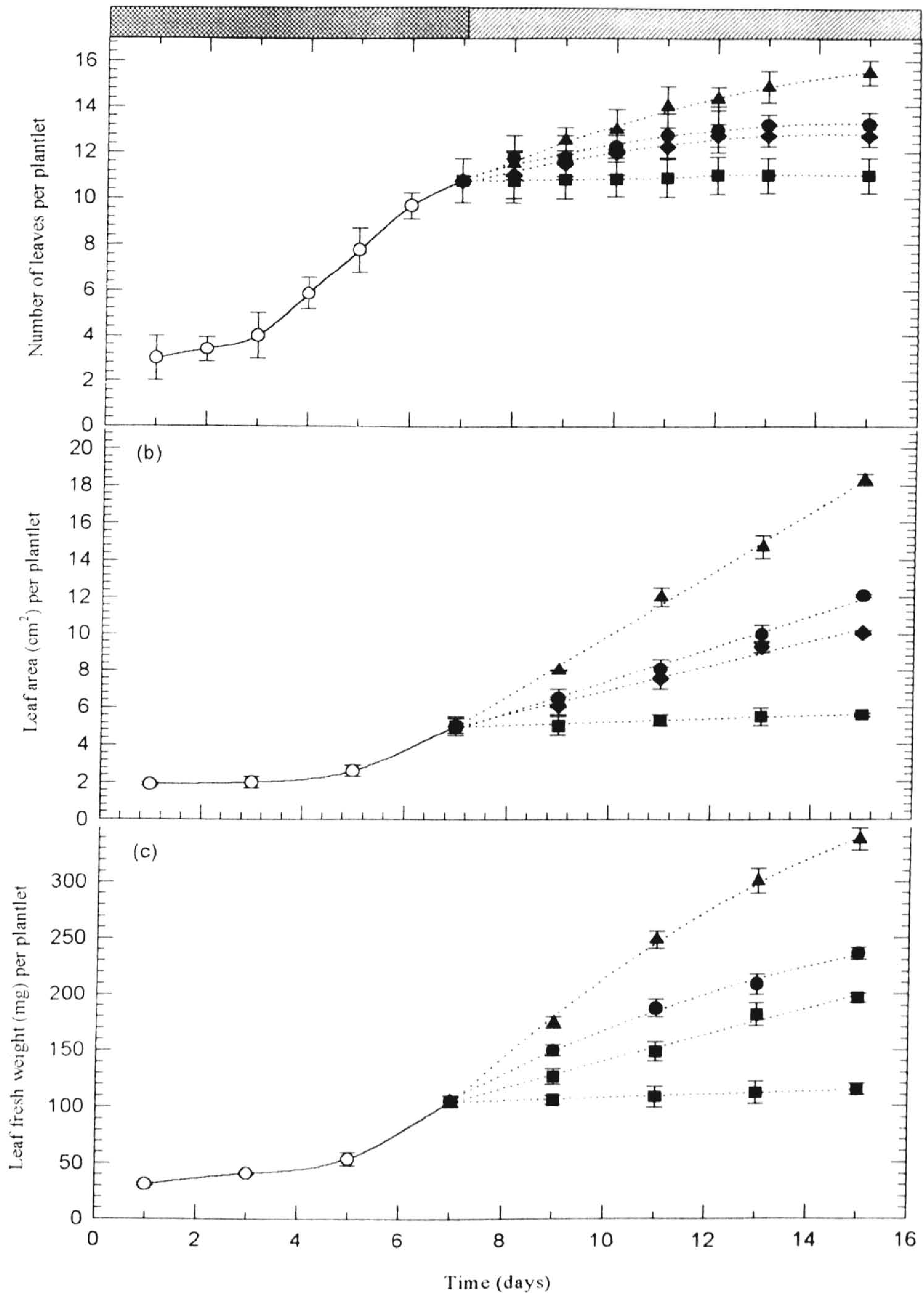



**Fig 5.15.** Effects of closed (■), diffusive (◆) and forced ventilation - slow flow ventilation (flow rate=1.0-1.5 $cm^3$ $min^{-1}$) (●); fast flow ventilation (flow rate=3.5 $cm^3$ $min^{-1}$) (▲); on (a) leaf number (b) leaf area ($cm^2$) and (c) leaf fresh weight (mg) of *in vitro* tobacco cuttings grown in 60 $cm^3$ glass vessels containing half strength MS medium (10 ml) lacking any hormone. (The plants had been previously grown 7 days in fast flow ventilation).

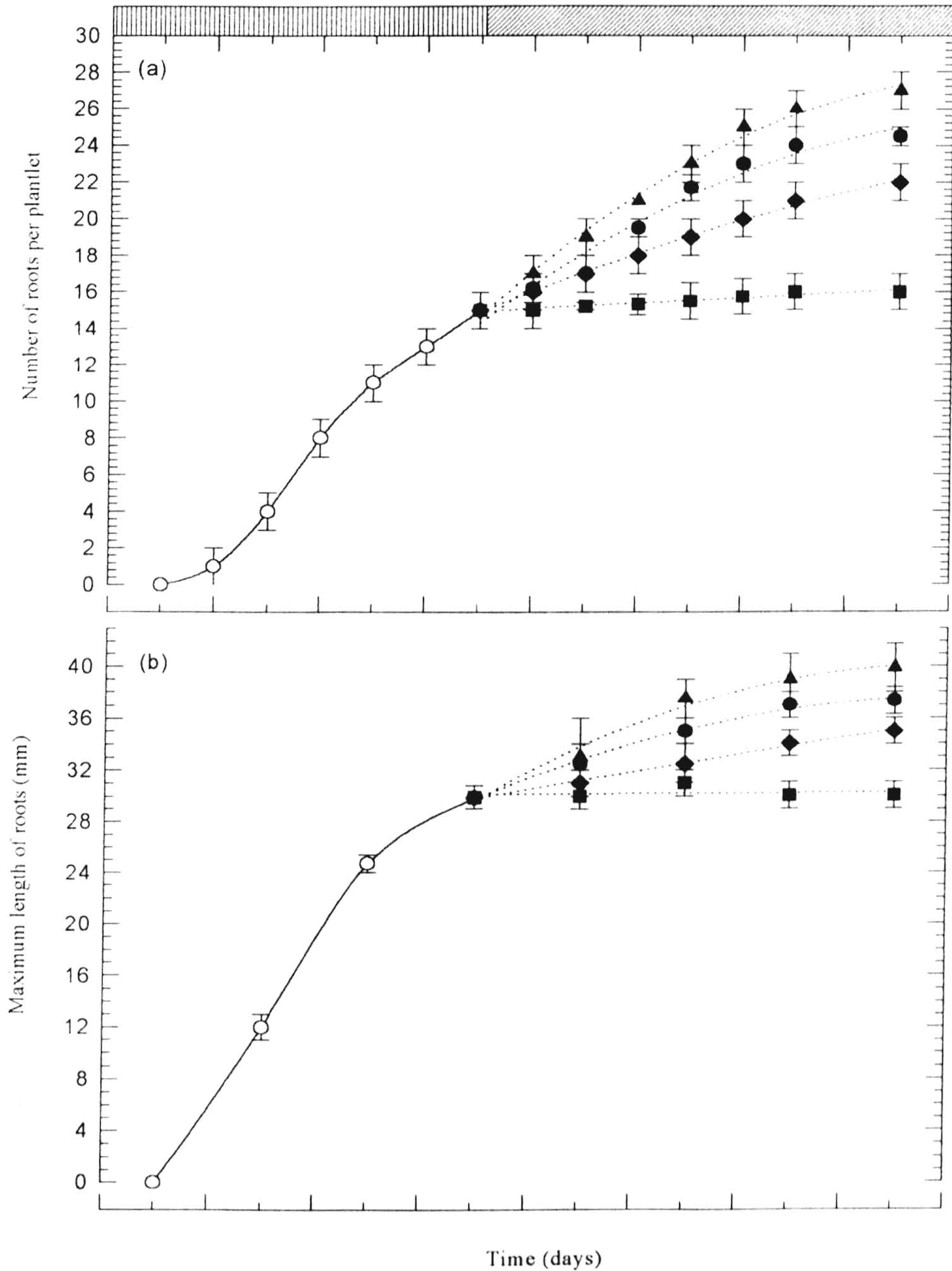



**Fig 5.16.** Effects of closed (■), diffusive (◆) and forced ventilation - slow flow ventilation (flow rate=1.0-1.5 $cm^3$ $min^{-1}$) (●); fast flow ventilation (flow rate=3.5 $cm^3$ $min^{-1}$) (▲); on (a) number of roots and (b) maximum length of roots (mean maximum root length, 3 roots from each plantlets) of *in vitro* tobacco cuttings grown in 60 ml glass vessels containing half strength MS medium (10 ml) lacking any hormone. (The plants had been previously grown 7 days in fast flow ventilation).

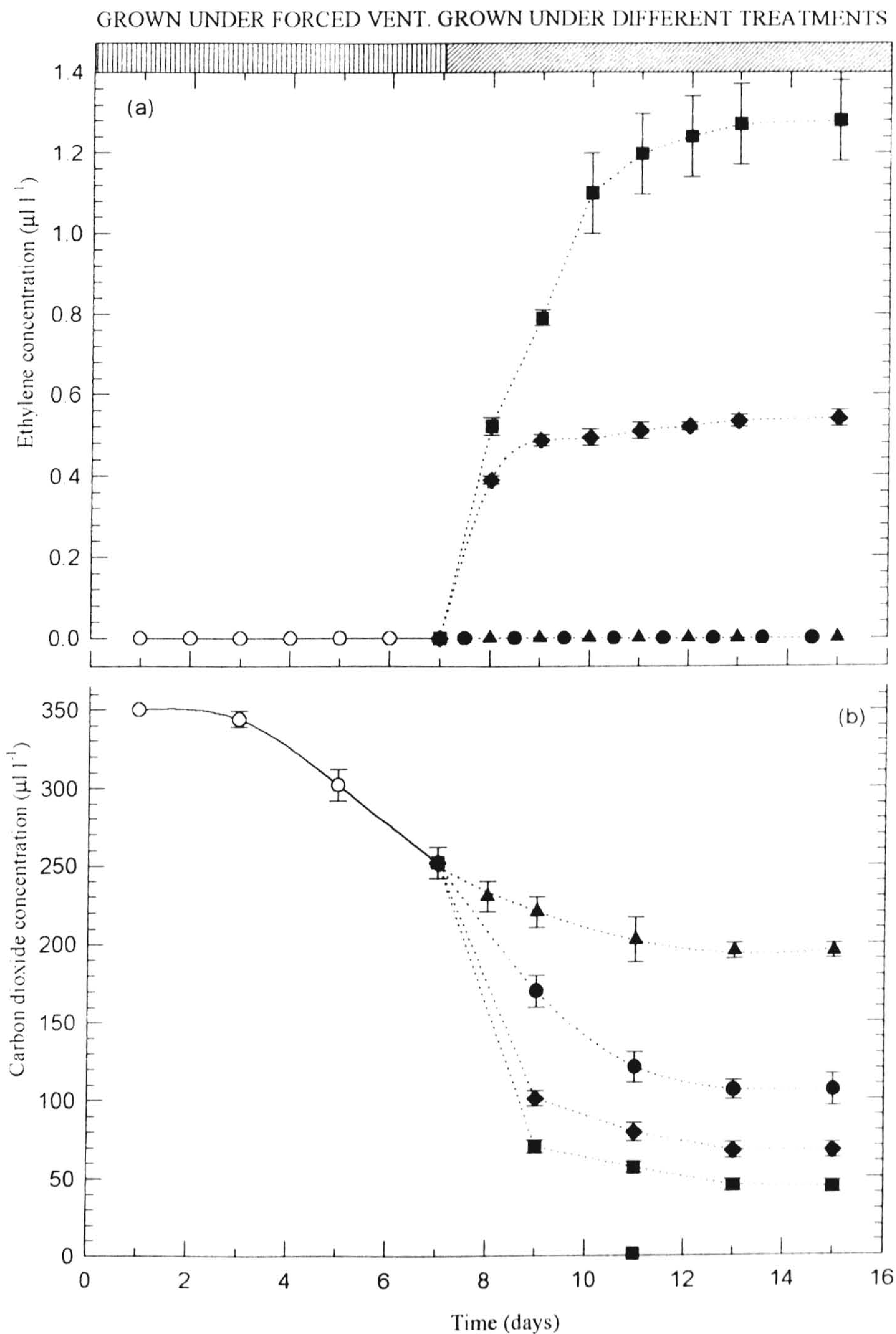



**Fig 5.17.** Effects of closed (■), diffusive (◆) and forced ventilation - slow flow ventilation (flow rate=1.0-1.5 $cm^3$ $min^{-1}$) (●); fast flow ventilation (flow rate=3.5 $cm^3$ $min^{-1}$) (▲); on (a) ethylene and (b) carbon dioxide concentration in the head-space of *in vitro* tobacco cuttings grown in 60 ml glass vessels containing half strength MS medium (10 ml) lacking any hormone. (The plants had been previously grown 7 days in fast flow ventilation).

44 $\mu l\ l^{-1}$, i.e. at or close to the $CO_2$ compensation point (Fig. 5.17b). With diffusive ventilation, $CO_2$ concentrations in the culture vessels were eventually reduced 67 $\mu l\ l^{-1}$; this was still 1.5X greater that of the sealed ones, and probably still above the $CO_2$ compensation point. With FF-ventilation the $CO_2$ concentrations had equilibrated at *ca.* 200 $\mu l\ l^{-1}$, and this was substantially higher than that of even the SF system (105 $\mu l\ l^{-1}$).

**5.3.5.3. Chlorophyll contents**

Total chlorophyll contents based on the fresh weight of leaves declined steadily in sealed vessels with the passage of time (Fig. 5.18), and was only 70 $\mu g\ g^{-1}$ fresh weight at the end of the experiment, a very low value. In contrast, plantlets grown under forced ventilation exhibited much higher chlorophyll contents (1337 $\mu g\ g^{-1}$ fresh weight). Chlorophyll content also decreased in the diffusive and SF ventilation treatments, but there were substantial differences between them and values with diffusive flow were <0.5X that in the SF system.

The conclusion drawn from the above results is that the yellowing of the leaves of tobacco plantlets which was observed mainly in the sealed and diffusive systems may be due to the higher ethylene concentration accumulated in the head-space of the culture vessels. The epinastic curvature of the leaves was evidence of that. However, to some extent the lower $CO_2$ concentration noticed in these two treatments might also be responsible since under SF-ventilation some leaves became yellow in colour but ethylene did not accumulate

The results obtained point to the need for further experiments in which ethylene should be applied to the FF-ventilation system to determine whether it will induce yellowing and reduce growth under conditions where $CO_2$ levels remain high and volatiles other than ethylene cannot accumulate. The results of such an experiment are reported in the next section.

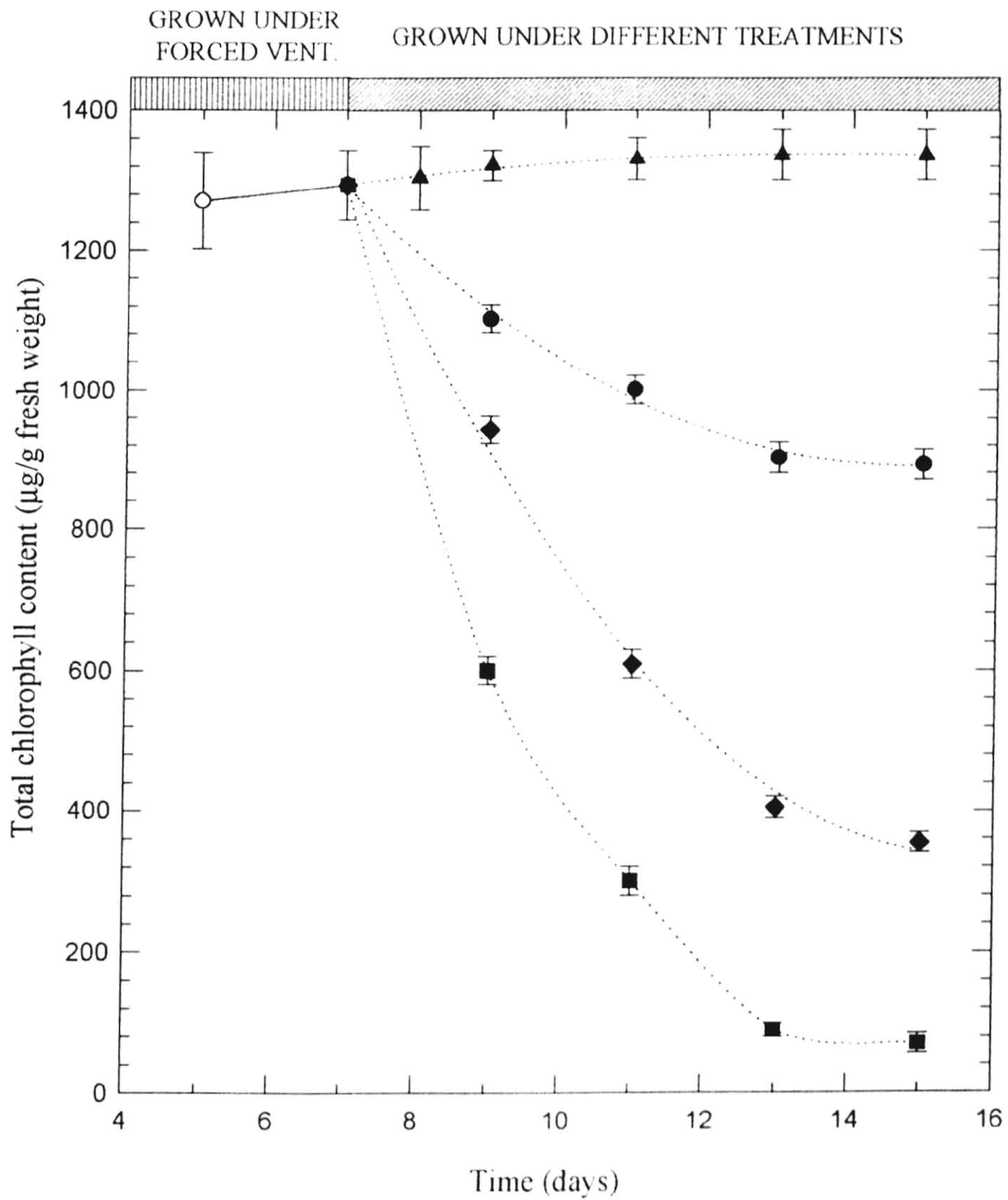



**Fig 5.18.** Effects of closed (■), diffusive (◆) and forced ventilation - slow flow ventilation (flow rate=1.0-1.5 $cm^3$ $min^{-1}$) (●); fast flow ventilation (flow rate=3.5 $cm^3$ $min^{-1}$) (▲); on chlorophyll contents (based on leaf fresh weight) of *in vitro* tobacco cuttings grown in 60 ml glass vessels containing half strength MS medium (10 ml) lacking any hormone. (The plants had been previously grown 7 days in fast flow ventilation).

### 5.3.6. Continuous exposure to ethylene under FF-ventilation

Plants were first grown under FF-ventilation and then, at 15 days, were subjected to either FF with ethylene (1.5-2.0 μl $l^{-1}$) or FF only (control). Of the two treatments the better growth subsequently occurred in the control (Table 5.05). After only 4-days both the area and fresh weights of leaves per plantlet in the controls were approx. 1.8X greater than those in the continuous ethylene treatment. The numbers of leaves and the stem fresh weights were the same from both treatments; root numbers and maximum lengths of roots were slightly higher in the controls. In the continuous ethylene with forced flow ventilation, the leaves went yellow within the four days of treatment and the young leaves were showing signs of epinasty, whereas in the controls the natural green colour of the leaves was largely maintained and there was no epinasty (Plate 5.06).

**TABLE 5.05. Effects of continuous exposure of ethylene (1.5 - 2.0 μl $l^{-1}$) under fast flow (FF) ventilation (5.0 $cm^3$ $min^{-1}$) on the growth of tobacco plantlets after 4 days (plantlets had been previously grown with fast flow ventilation for 15 days).**

| Treatments | Leaves | | | Stem | Root | |
|---|---|---|---|---|---|---|
| | No. | Area ($cm^2$) | Fresh weight (mg) | Fresh weight (mg) | No. | †Max. Length (mm) |
| Control (FF-ventilation) | 9.0±0.6 | 21.4±1.0 | 758.8±60.8 | 38.3±0.5 | 20.3±1.0 | 68.1±1.5 |
| FF-ventilatio+ Ethylene (1.5-2.0μl $l^{-1}$) | 8.7±0.3 | 11.8±0.3 | 427.4 ± 13.1 | 39.3±0.6 | 18.0±0.7 | 58.3±1.76 |

†mean maximum root length (3 roots from each seedlings);
*cultures were grown in continuous light condition (150 μmol $m^{-2}$ $s^{-1}$).

The chlorophyll contents based on the fresh weights of leaves were substantially lowered in the continuous ethylene treatment. In contrast much higher chlorophyll contents (a and b) and carotenoids were recorded in the controls: respectively 4.6X, 8.2X and 5.5X greater than in the continuous ethylene treatment (Table 5.06).

## PLATE : 5.06

Tobacco cuttings *in vitro*: effects of continuous exposure to ethylene (1.5 - 2.0 $\mu l\ l^{-1}$ in air) with fast flow ventilation (5 $cm^3\ min^{-1}$) after 5 days.

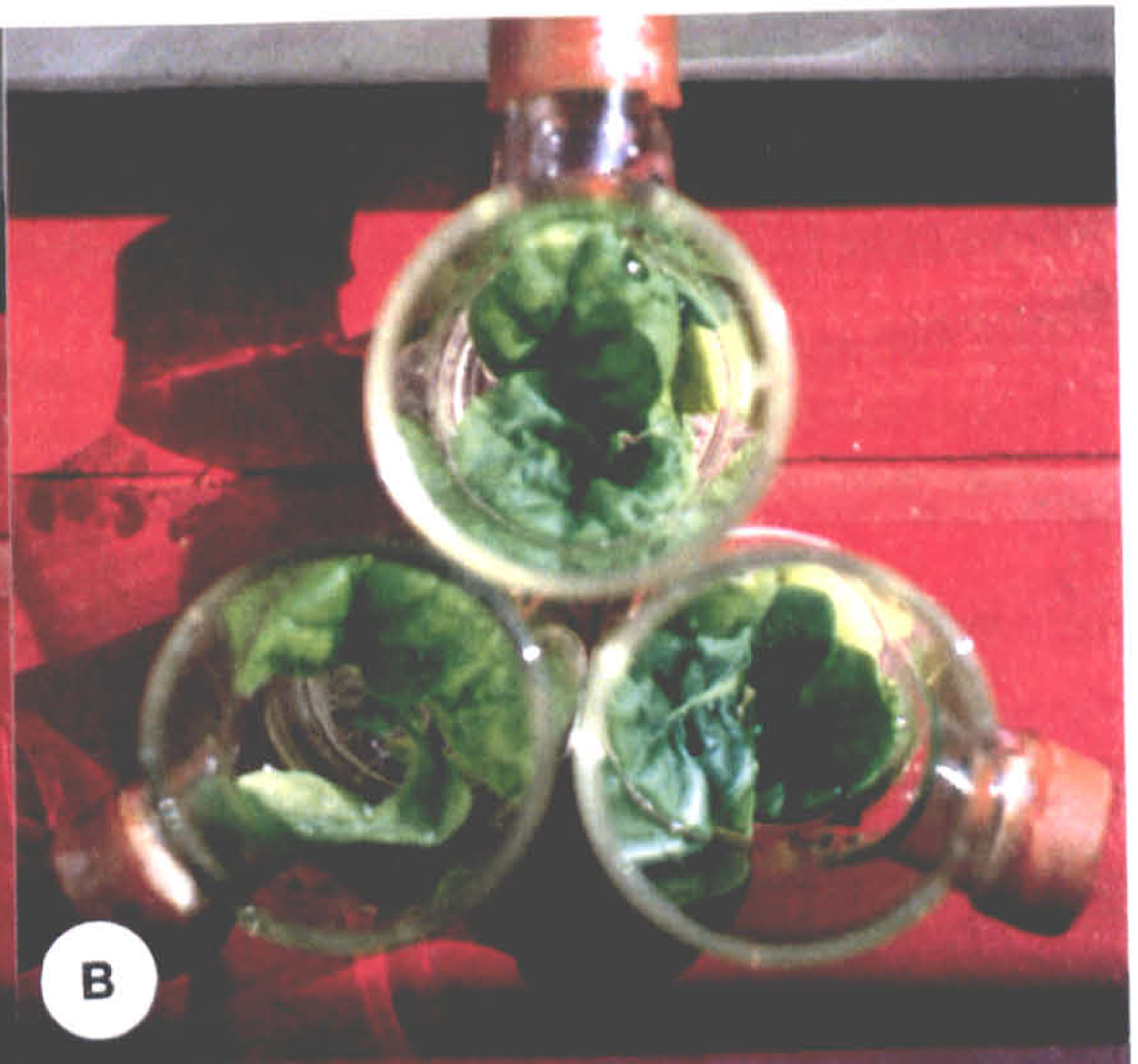
The plants had been previously grown for 15 days in 60 ml glass vessels containing half strength MS medium (10 ml) lacking any hormone and with fast flow ventilation.

A, C, and E: The inflow turrets were inserted into a 'wind-tunnel' (Fig. 5.02) through which the ethylene mixture was passed (speed = 3.7 $ms^{-1}$). Note the leaves became very yellow from the base upwards (X1.4).

B, D and F: Here the inflow turrets were in the air. Note leaves remained healthy and mostly green (X1.4).

Growth room conditions: T = 25°C and continuous light (PAR =150 $\mu mol\ m^{-2}\ s^{-1}$).

A


B

C

D

E

F

These results reveal that ethylene can indeed exert very deleterious effects on tobacco at concentrations which might readily occur in sealed and diffusively ventilated culture systems. They lend support to the view that it probably exerted significant effects in most of the experiments reported in this chapter.

**TABLE 5.06. Effects of continuous exposure of ethylene (1.5 - 2.0 μl $l^{-1}$) under fast flow (FF) ventilation (5.0 $cm^3$ $min^{-1}$) on chlorophyll and carotenoid contents of leaves of tobacco plantlets after 4 days (plantlets had been previously grown with fast flow ventilation for 15 days).**

| Characteristics | Control (FF- ventilation) | FF- ventilation + Ethylene (1.5 - 2.0 μl $l^{-1}$) |
|---|---|---|
| **Chlorophyll a** μg(g fresh wt)$^{-1}$ | 1228.6 ± 62.3 | 144.8 ± 10.3 |
| **Chlorophyll b** μg(g fresh wt)$^{-1}$ | 268.6 ± 12.3 | 17.6 ± 2.1 |
| **Carotenoids contents** μg(g fresh wt)$^{-1}$ | 313.8 ± 19.3 | 56.7±5.3 |

*cultures were grown in continuous light condition (150 μmol $m^{-2}$ $s^{-1}$).

## 5.4. FINAL COMMENTS

The results have shown that the growth of tobacco plantlets can be substantially improved by means of forced ventilation of the culture vessels and in general the best growth was achieved with the higher flow. In sealed vessels the growth was extremely retarded probably because of (a) $CO_2$ deficiency in the culture atmosphere and (b) an accumulation of ethylene. The results from ethylene inhibitors did not show any clear evidence of an ethylene effect, but the ACC additions and the ACC plus silver do suggest that ethylene could have been important. Moreover, the continuous exposure of plantlets to exogenous ethylene (1.5 - 2.0 $\mu l\ l^{-1}$) under FF-ventilation revealed that ethylene can severely depress the growth of tobacco plantlets. Aharoni and Lieberman (1979) pointed out that in tobacco endogenous ethylene plays a considerable role in the regulation of leaf senescence. Endogenous ethylene within mature leaves of tobacco range between 0.1 and 0.2 $\mu l\ l^{-1}$ (Aharoni, 1978; Aharoni and Lieberman 1979) and threshold values for an ethylene effect on leaf senescence were reported to be 0.01 to 0.1 $\mu l\ l^{-1}$. In the present investigation the accumulation of endogenous ethylene in the culture atmosphere varied from 0.03 to 0.54 $\mu l\ l^{-1}$ in diffusive ventilation and 0.2 to 1.2 $\mu l\ l^{-1}$ under sealed conditions. The flushing out of the accumulated ethylene from the culture vessels solved this problem and thus was partially responsible for the better growth compared with that in the sealed or diffusive treatments. However, the ability of the forced ventilation systems to improve $CO_2$ concentrations in the culture vessels might have had large effects in producing better growth in tobacco.

It is already well known that ethylene and ethylene-releasing chemicals have been used to attain a bright yellow colour and to reduce the curing time of tobacco leaves (Abeles 1973). The ethylene-induced chlorophyll loss has been also reported in orange (Shamoute orange) and other fruits (Apelbaum, Goldschmidt and Yehoshua 1976) and also in leaves of tobacco (Burg 1968, Pratt and Goeschl 1969). In the present investigation, tobacco leaves became yellow in the sealed vessels and in the forced ventilation with added ethylene but normal $CO_2$ concentrations. Thus, accumulated

endogenous ethylene was possibly a more immediate cause of senescence and epinasty in sealed vessels than any lack of $CO_2$. The chlorophyll was also depleted substantially in the diffusively ventilated vessels and SF-ventilation showed some signs of yellowing but not to a great extent. Although in the case of the SF-ventilation system no ethylene was detected in the head-space, this does not rule out some slight endogenous inhibitory effect. However, the fast flow ventilation system *per se* was able to maintain the natural green colour of the leaves in tobacco plantlets.

Therefore, considering all the factors represented in this chapter, it could be strongly recommended that forced ventilation is necessary for the substantial growth and long term survival (up to three to four weeks) of *in vitro* grown tobacco cultures.

# CHAPTER VI

## LEAF ANATOMY OF TOBACCO AND CAULIFLOWER PLANTLETS: EVALUATION OF CLOSED SYSTEM, DIFFUSIVE AND FORCED VENTILATION

### 6.1. INTRODUCTION

Poor survival rates of plantlets during the period of acclimatisation can greatly limit the application of micropropagation techniques (Marin, Gella and Herrero 1988). The reasons for this difficulty include reduced amounts of epicuticular wax (Grout 1975; Sutter and Langhans 1982), poor cuticle development and improperly functioning stomata (Brainerd and Fuchigami 1981, 1982; Fuchigami, Cheng and Soeldner 1981) which results in excessive water loss, poor photosynthetic capacity (Donnelly, Vidaver and Colbox 1984, Grout and Millam, 1985) and anatomical abnormalities (Wetzstein and Sommer 1982).

Investigations have also been made to see whether leaves were modified internally as a consequence of developing in the culture environment. Comparisons were made between leaves produced in culture, leaves produced by the *in vitro* grown plantlets after transplanting and those of greenhouse-grown seedlings (Wetzstein and Sommer 1982; Grout and Aston 1978). In all these investigations it was revealed that in regenerated plants normal structure and function of stomata did not appear until one to several months after removal from culture.

The aim of the present study was to see if internal leaf anatomy, epicuticular waxes and stomatal function of tobacco and cauliflower plantlets is improved by introducing forced ventilation in the culture vessels. To this end plantlets grown under forced ventilation have been compared with those produced in sealed conditions, in diffusively ventilated vessels and *in vivo*.

## 6.2. MATERIALS AND METHODS

### 6.2.1. Plant material

Aseptically grown cauliflower (*Brassica oleracea* var. botrytis L.) and tobacco (*Nicotiana tabacum*, White Burly) seedlings were used as experimental material. Shoot tips from 5 days old seedlings were cut into 4-5 mm lengths and inoculated on to the medium.

### 6.2.2. Establishment of culture

Half strength MS (Murashige and Skoog 1962) medium was used as the basal medium. Glass tubes (60 $cm^3$) with a side arm were used as culture vessels, each of which contained 10 ml of medium. The side arm was sealed with a silicone rubber 'Suba-seal'. Each vessel was capped with either a) a silicone rubber bung to seal the container or b) a disc of polypropylene membrane for diffusive ventilation or c) a single FF-ventilation apparatus for *fast* forced ventilation (flow rate = 5.0 $cm^3$ $min^{-1}$) or d) two sets of FF-ventilation apparatus arranged in parallel (flow rate = 9.0-10.0 $cm^3$ $min^{-1}$) for very fast forced ventilation. Plants grown in soil under normal growth room conditions were employed as an *in vivo* control Cultures were incubated at *ca.* 25°C with 8 hour dark and 16 hour light periods (PAR = 150 μmol $m^2$ $s^{-1}$).

### 6.2.3 Measurements of ethylene and $CO_2$ concentrations and RH

These were performed according to methods described in Chapter IV: Section 4.2.4.1. The ethylene and $CO_2$ concentrations and percentage of RH of the head-spaces of the culture vessels were measured every 7 days at the end of light period. The experiments ran for 28 days.

### 6.2.4. Anatomy

Unless otherwise stated the anatomical studies were conducted on plants which had been cultured for 4 weeks

#### 6.2.4.1. Light microscopy: stomatal measurements and cuticular waxes

For studying stomata, the lower epidermises of the fresh third or fourth leaf from the apex of tobacco and cauliflower plants were prepared in one of two ways and examined by light microscopy: *either* (a) epidermal peels were taken from the abaxial (lower) surfaces of the leaves, mounted in water and then studied and photographed using a precalibrated microscope (Olympus BX40), *or* (b) the lower epidermis was examined from peels or from entire pieces of leaf, stained in 0.02% aqueous auramine and photographed under blue light to show waxes fluorescing yellow (using a Zeiss photo microscope).

Densities (no. per $mm^2$), lengths and widths of stomata (with guard cells) and pore dimensions were measured, directly under the microscope, for each treatment, using method (a). Areas of stomatal pores and of entire stomata, relative to leaf areas were measured from photographs according to methods (a) and (b), by tracing and the use of a leaf area meter (Lambda Instruments Corp.).

For studying epicuticular waxes hand-cut transverse sections of fresh leaves were stained in auramine and examined as in (b)

#### 6.2.4.2. Resin embedding

Samples of leaves (2 X 5 mm pieces) were first preserved in 6% glutaraldehyde overnight at 4°C (pH 6.8); this was then replaced with 0.05 M Cacodylate buffer to help the fixative to penetrate the tissues. The leaf samples were then transferred to 1% osmium tetroxide fixative for 4 hours, washed several times with distilled water, and then dehydrated, first using 30% ETOH followed by a gradual increase in percentage, and finally with 100% ETOH. The samples were next placed in a mixture of 100% ETOH and LR white resin for 3 hours, then embedded in LR white resin (medium grade acrylic resin) in Beem capsules, and finally incubated at 60°C for 2-3 days. Transverse sections were obtained using an ultramicrotome (Reichart OMU2) with a glass knife. The sections (2-4 µm) were stained with toluidine blue (1% w/v in 1% Borax solution) and examined by light microscopy.

### 6.2.4.3. Scanning electron microscopy

Cauliflower leaf stomata were also examined by SEM from plants grown under sealed, diffusive, slow flow ventilation (1.0 $cm^3$ $min^{-1}$) and fast flow ventilation (5.0 $cm^3$ $min^{-1}$) for 15 days. Small leaf samples (*ca.* 2 X 5 mm), from the third or fourth leaf from the apex were taken from the plant and immediately placed into fixative: a mixture of 3% glutaraldehyde and 15% paraformaldehyde in phosphate buffer (pH 6.8) for 5 days. This was followed by dehydration in acetone. Finally they were dried by the $CO_2$ critical-point drying technique, and coated in gold. The lower epidermal surfaces were examined by SEM.

## 6.3. RESULTS AND DISCUSSION

### 6.3.1. Culture atmosphere

#### 6.3.1.1. Relative humidity

The RH values of the culture vessels subjected to sealed and diffusive ventilation were significantly higher than in the other treatments (Fig. 6.01). In sealed as well as in the diffusive treatments, nearly 100% RH was observed in the culture vessel atmospheres throughout the experiment with both cauliflower and tobacco. In contrast, with normal fast flow ventilation, the RH recorded in the culture atmosphere for both species was initially *ca.* 88%, but by the end of the experiment (4 weeks) the values had risen to around 97%. With very fast flow ventilation, however, the initial RH values were lower, *i.e.* only *ca.* 75% after 2 weeks, and for both species had increased only to 92% by the end of the experiment.

The rapid increases in RH after 2 weeks with forced ventilation were no doubt because the plants were becoming larger and the transpiration rates higher

#### 6.3.1.2. Ethylene concentration

In sealed containers ethylene concentrations increased rapidly and during light periods reached peak levels of 1.4 $\mu l\ l^{-1}$ in cauliflower and 1.05 $\mu l\ l^{-1}$ in tobacco by the end of the experiment (Fig. 6.02). During the dark period the concentrations decreased by *ca.* 30% for both species (data not shown).

Compared to the sealed treatments the ethylene concentrations during light periods under diffusive ventilation were significantly lower, *ca.* 0 1 $\mu l\ l^{-1}$ for both species. With both fast and very fast forced ventilations no ethylene accumulation was recorded in the culture vessels.

Although ethylene accumulation was much less in the diffusive treatment compared to the sealed system it is interesting that the accumulation was more evident after 15 days rising in parallel with the relative humidity, and presumably plant size.

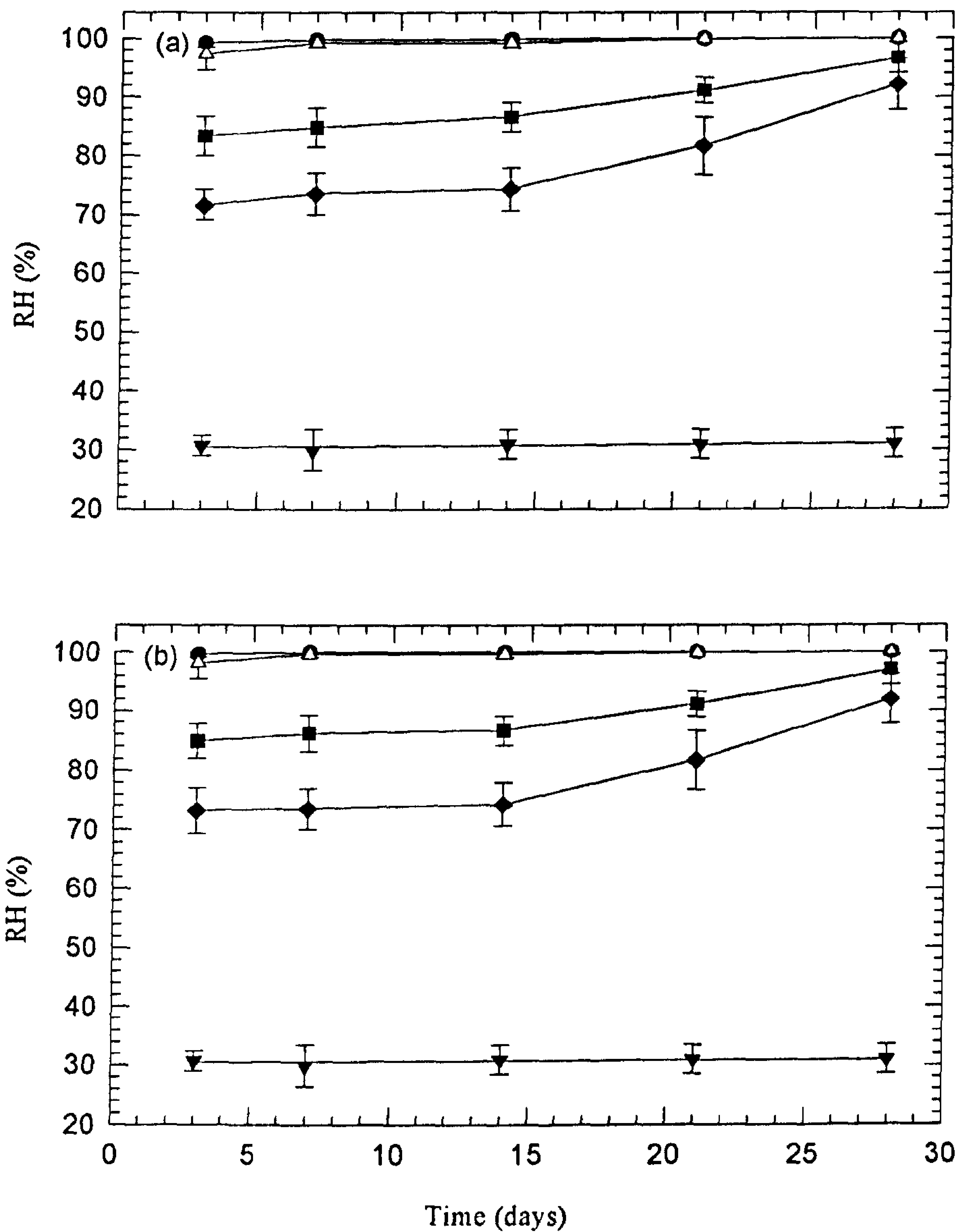



**Fig. 6.01.** Showing the percentage of relative humidity in the culture head-space during the light period of (a) cauliflower plantlets and (b) tobacco plantlets grown *in vitro* under different types of ventilation; closed (each vessel was sealed with a silicone rubber bung ●), diffusive ventilation (each vessel was capped with a polypropylene disc Δ), fast forced ventilation (each vessel was fitted with a single ventilation apparatus) - flow rate was 5 $cm^3$ $min^{-1}$ ■, very fast forced ventilation (each vessel was fitted with two sets of ventilation apparatus) - flow rate was 10 $cm^3$ $min^{-1}$ ◆) and *in vivo* (growth room conditions▼). Cultures were grown at *ca.* 25°C with 8 hour dark and 16 hour light periods; PAR = 150 µmol $m^{-2}s^{-1}$. Volume of culture vessel = 60 $cm^3$. Each bar represents a mean ± SE of 5 replicates.

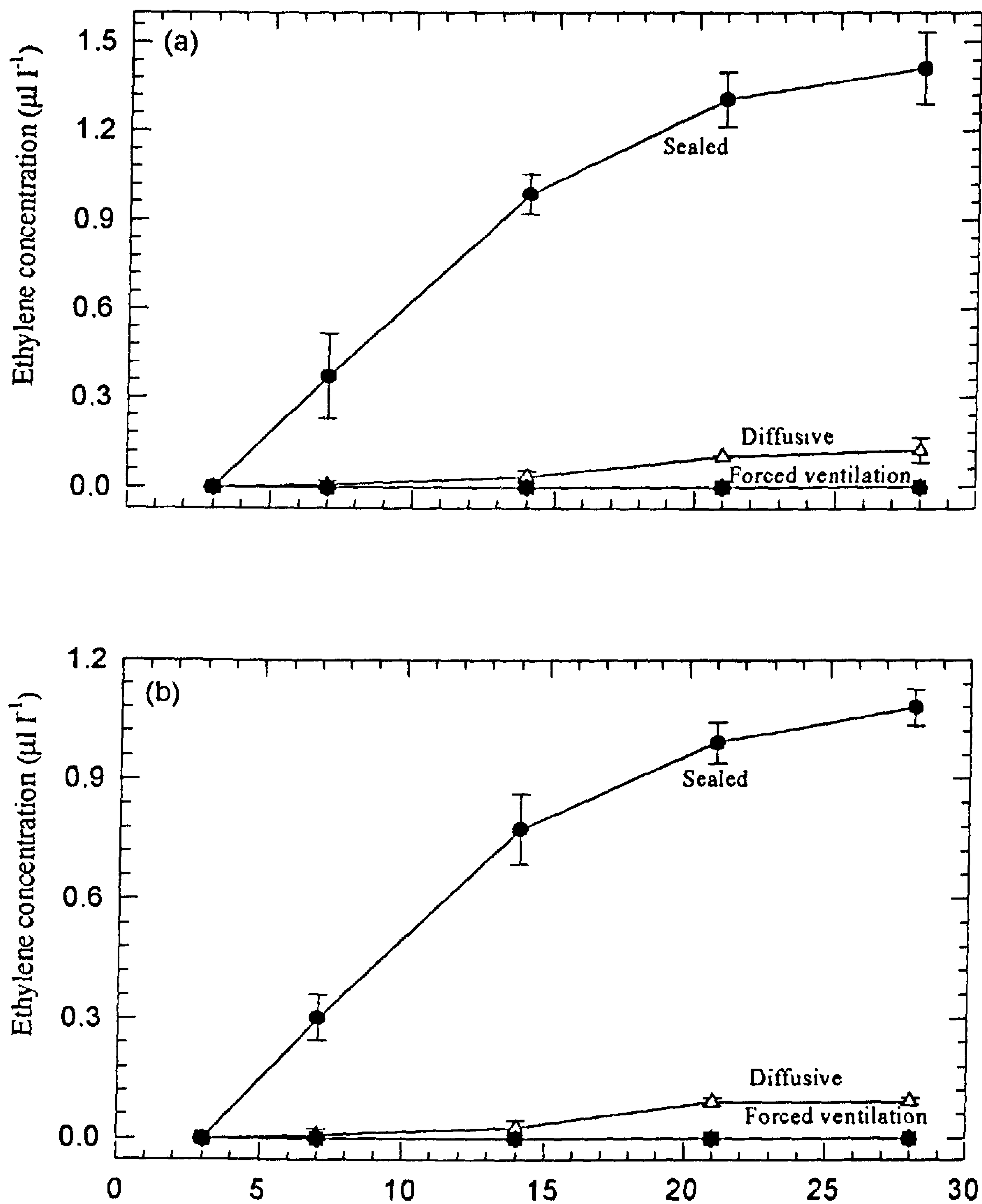



**Fig. 6.02.** Showing ethylene concentration in the culture head-space during the light period of (a) cauliflower plantlets and (b) tobacco plantlets grown *in vitro* under different types of ventilation; closed (each vessel was sealed with a silicone rubber bung ●), diffusive ventilation (each vessel was capped with a polypropylene disc Δ), fast forced ventilation (each vessel was fitted with a single ventilation apparatus) - flow rate was 5 $cm^3$ $min^{-1}$ ■, very fast forced ventilation (each vessel was fitted with two sets of ventilation apparatus) - flow rate was 10 $cm^3$ $min^{-1}$ ◆) and *in vivo* (growth room conditions▼). Cultures were grown at *ca.* 25°C with 8 hour dark and 16 hour light periods; PAR = 150 µmol $m^{-2}$ $s^{-1}$. Volume of culture vessel = 60 $cm^3$. Each bar represents a mean ± SE of 5 replicates.

### 6.3.1.3. Carbon dioxide concentration

For both cauliflower and tobacco in the sealed condition $CO_2$ concentrations in the culture vessels measured during the photoperiod had decreased to *ca.* 65 $\mu l\ l^{-1}$ after 14 days, and finally reached 40 $\mu l\ l^{-1}$ after 28 days (Fig 6.03). In contrast, during darkness $CO_2$ concentrations increased and reached levels of *ca.* 1.4% at the end of the dark periods (4th week -data not shown).

Under diffusive ventilation the concentrations of $CO_2$ decreased down to 100 and 130 $\mu l\ l^{-1}$ after 14 days, and to 88 and 109 $\mu l\ l^{-1}$ after 28 days in cauliflower and tobacco respectively during the light periods.

Under fast flow ventilation $CO_2$ concentrations measured in the light remained high (>320 $\mu l\ l^{-1}$) for 14 days. Thereafter they decreased, but only to *ca.* 220 $\mu l\ l^{-1}$. At night the higher concentration of respiratory $CO_2$ was also controlled by the apparatus and the highest concentration at the end of the dark period was *ca.* 650 $\mu l\ l^{-1}$ in both the species (data not shown). In vessels with very fast flow ventilation, light period $CO_2$ concentrations remained high and close to atmospheric (325 $\mu l\ l^{-1}$) for both species, even after 28 days. During the dark periods concentrations were slightly in excess of atmospheric at *ca.* 360 - 400 $\mu l\ l^{-1}$, (data not shown).

## 6.3.2. Characteristics of stomata from lower epidermis of leaves

### 6.3.2.1. Arrangement and density

A noticeable feature of leaf stomata of both cauliflower and tobacco plants grown under the sealed condition and in diffusive ventilation was that they were so densely arranged that they were sometimes found adjacent to one another. (Plates 6.01, 6.02). This was not the case in the other treatments.

As shown in Fig 6.04 and Fig 6.05, the density of stomata in both the species, especially in the cauliflower, was significantly higher in plantlets subjected to sealed and diffusive treatments, and densities decreased with increasing efficiency of ventilation. With very fast flow ventilation densities were 0.32X and 0.63X the values for the sealed

## PLATE : 6.01

**Cauliflower :** Shoot cuttings and stomata from leaves after 28 days; culture vessel volume was 60 $cm^3$. Growth room conditions : *ca.* 25°C with 8 hours dark and 16 hours light periods; PAR = 150 $\mu mol\ m^{-2}s^{-1}$; RH = 26 - 32%.

(A - E) Stomata of lower epidermis of 3rd or 4th leaf from apex from dark period; plantlets were grown under different types of ventilation and also *in vivo* condition; slivers of lower epidermis were taken to show stomata (X468).

(A) sealed condition (silicone rubber bung); note that relatively larger stomata were densely arranged and gaping widely open .

(B) diffusive ventilation (polypropylene disc), note that large stomata were less densely arranged than above and also gaping widely open .

(C) fast forced ventilation (flow rate = 5 $cm^3\ min^{-1}$); note that slightfy smaller stomata were less densely arranged and stomatal pores were narrower in width than in (A) and (B).

(D) very fast forced ventilation (flow rate = 10 $cm^3\ min^{-1}$ ); note that stomata were smaller, less densely arranged and stomatal pores were narrower in width than in (A), (B) and (C).

(E) *in vivo* (growth room conditions); note low density of stomata which were much smaller in size with very narrow stomatal pore width compared to (A), (B) (C) and (D). The majority of the stomata were closed.

(F - I) Plantlets grown under different types of ventilation; (X1.2):

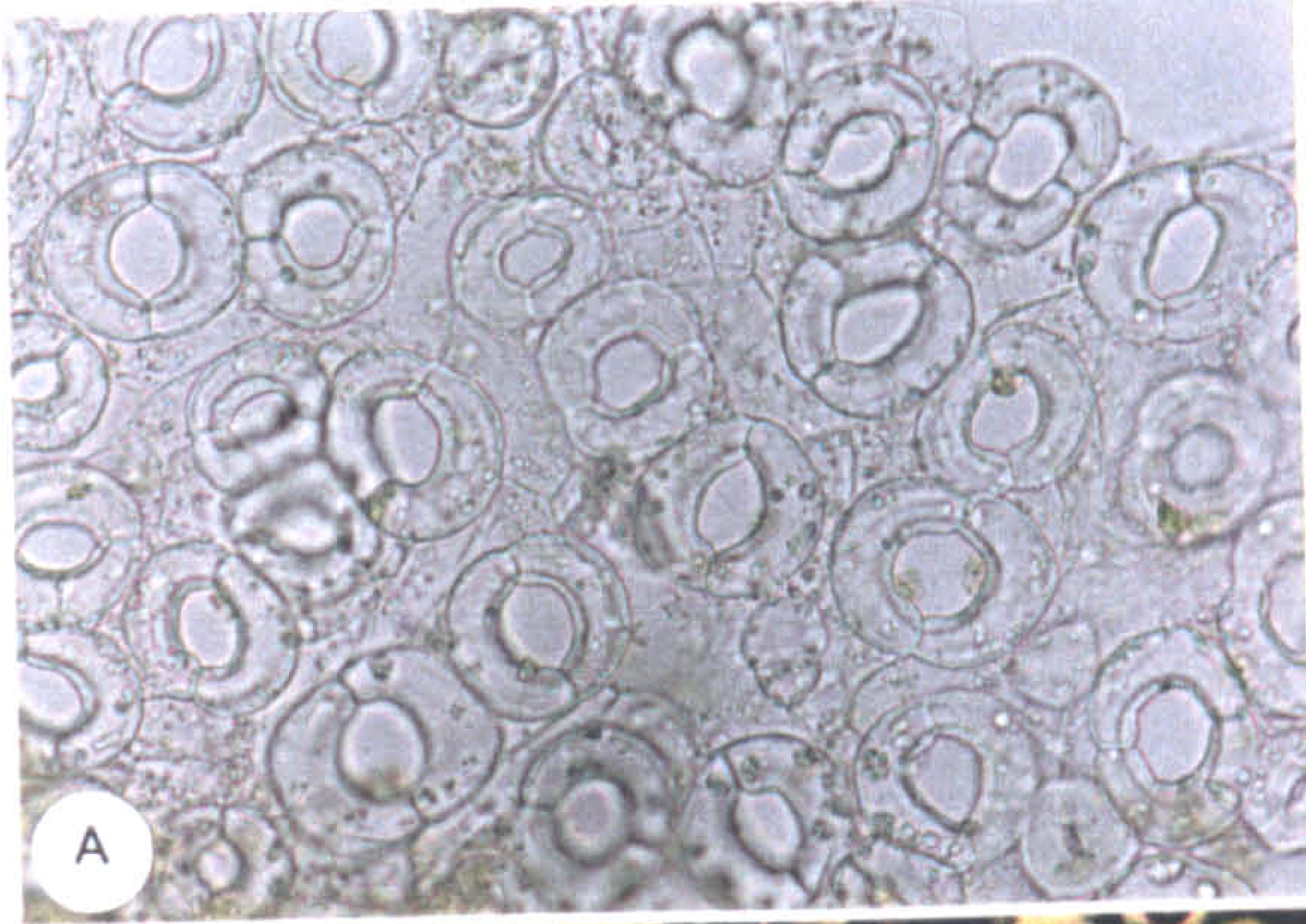
(F) sealed condition (silicone rubber bung); note leaves with very small area and showing epinastic curvature; some leaves had dropped. The root system was very poor in this treatment.

(G) diffusive ventilation (polypropylene disc), note leaves of moderate area and showing epinastic curvature; root system better developed than in (F).

(H) fast forced ventilation (flow rate was 5 $cm^3\ min^{-1}$); note larger leaf area than in (F) and (G) and absence of epinasty. Leaves looked blue-green due to the presence of epicuticular wax (evident as a white powdery coating). Root system better developed than in (F) and (G).

(I) very fast forced ventilation (flow rate was 10 $cm^3\ min^{-1}$); note even larger leaf area, absence of epinasty and better root development than in (H). Leaves looked more blue-green and had more epicuticular wax (evident as a white powdery coating) than in (H).

*In vivo* grown plants (not shown) were similar to (H) but larger (X1.5) and more and larger leaves.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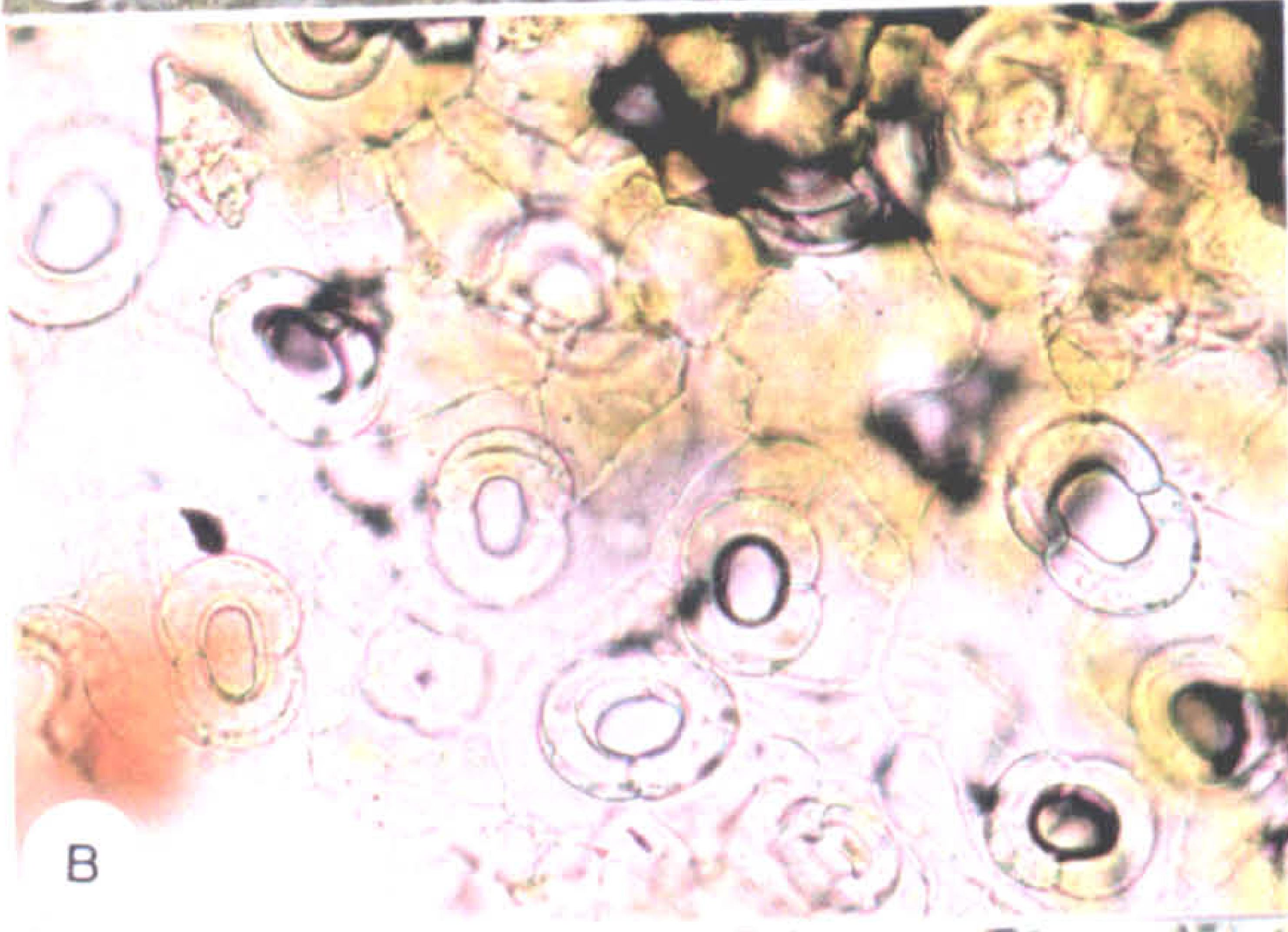
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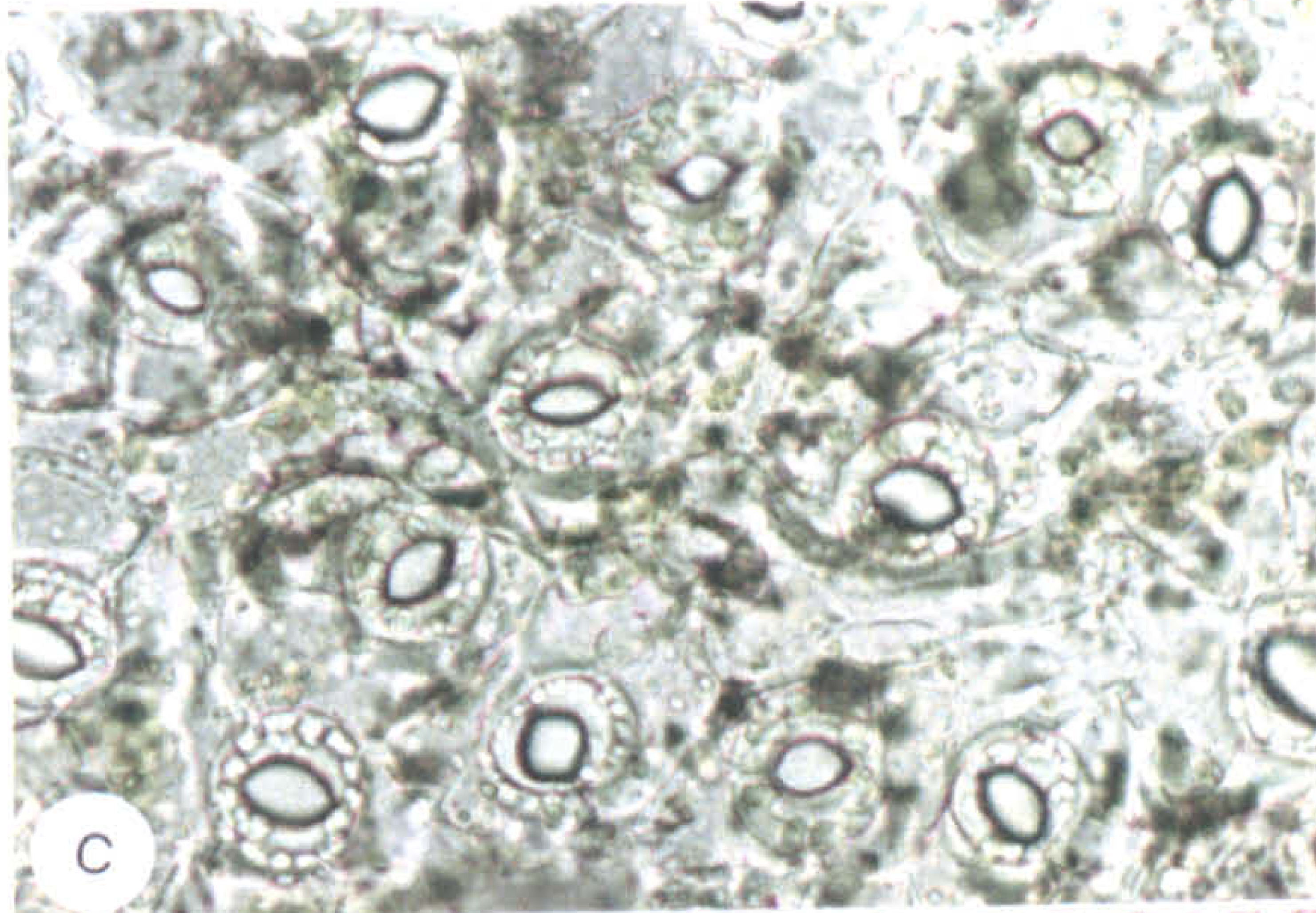
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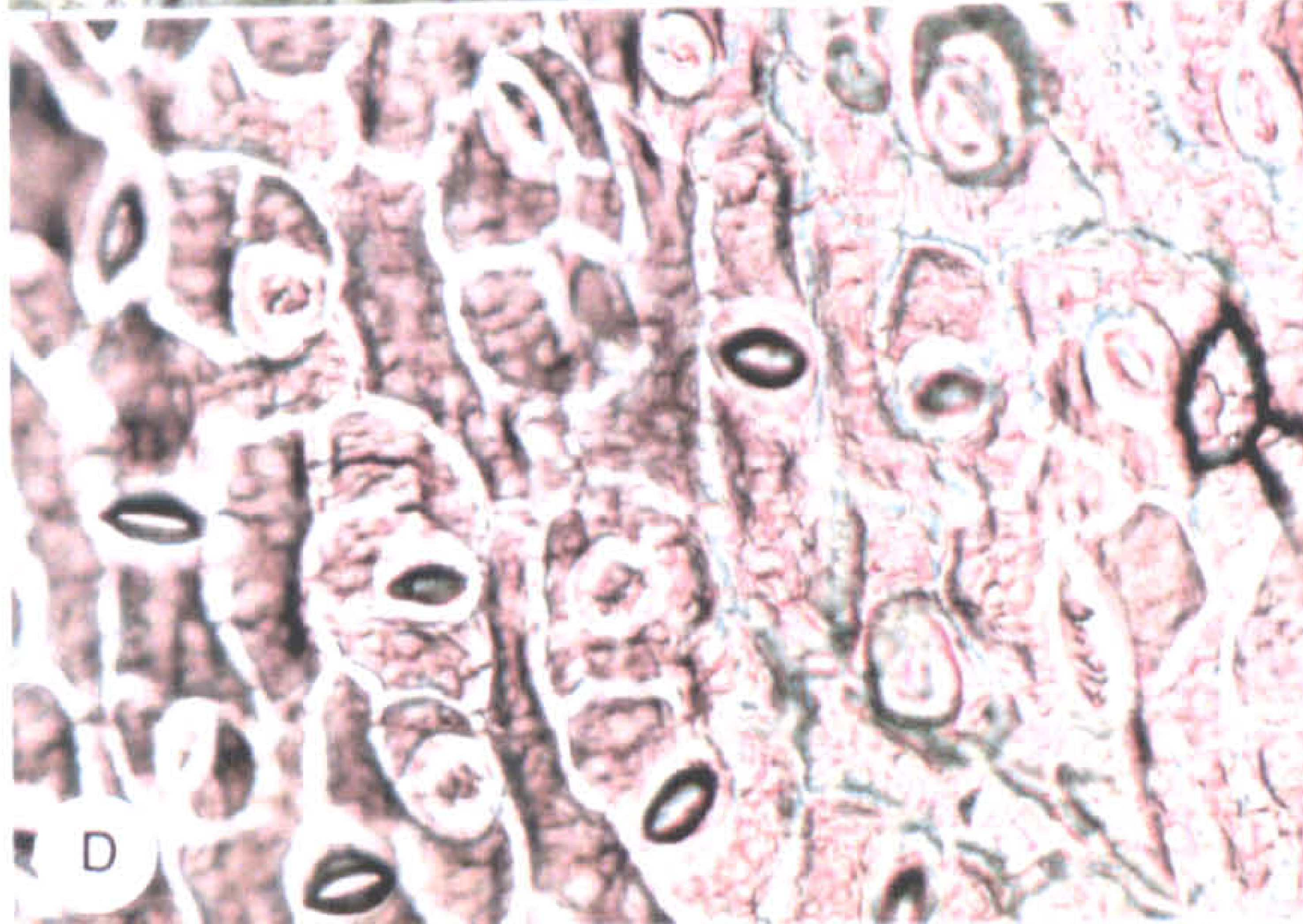
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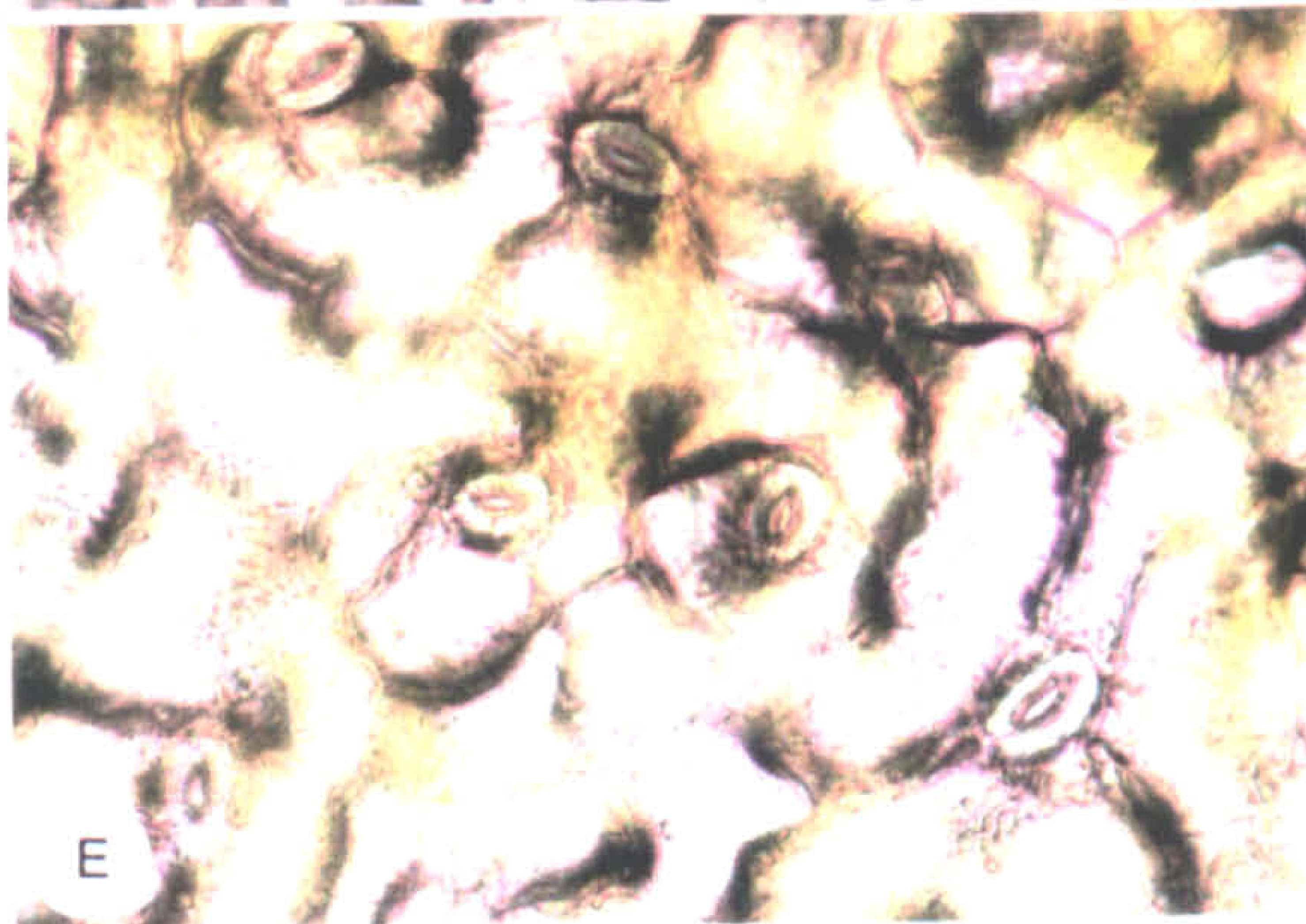
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F

G

H

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## PLATE : 6.02

**Tobacco :** Stomata from leaves of shoot cuttings after 28 days; culture vessel volume was 60 $cm^3$. Growth room conditions : *ca.* 25°C with 8 hours dark and 16 hours light periods; PAR = 150 µmol $m^{-2}s^{-1}$; RH = 26 - 32%.

Stomata of lower epidermis of 3rd or 4th leaf from apex from dark period; plantlets were grown under different types of ventilation and also *in vivo*; slivers of lower epidermis were taken to show stomata (X468).

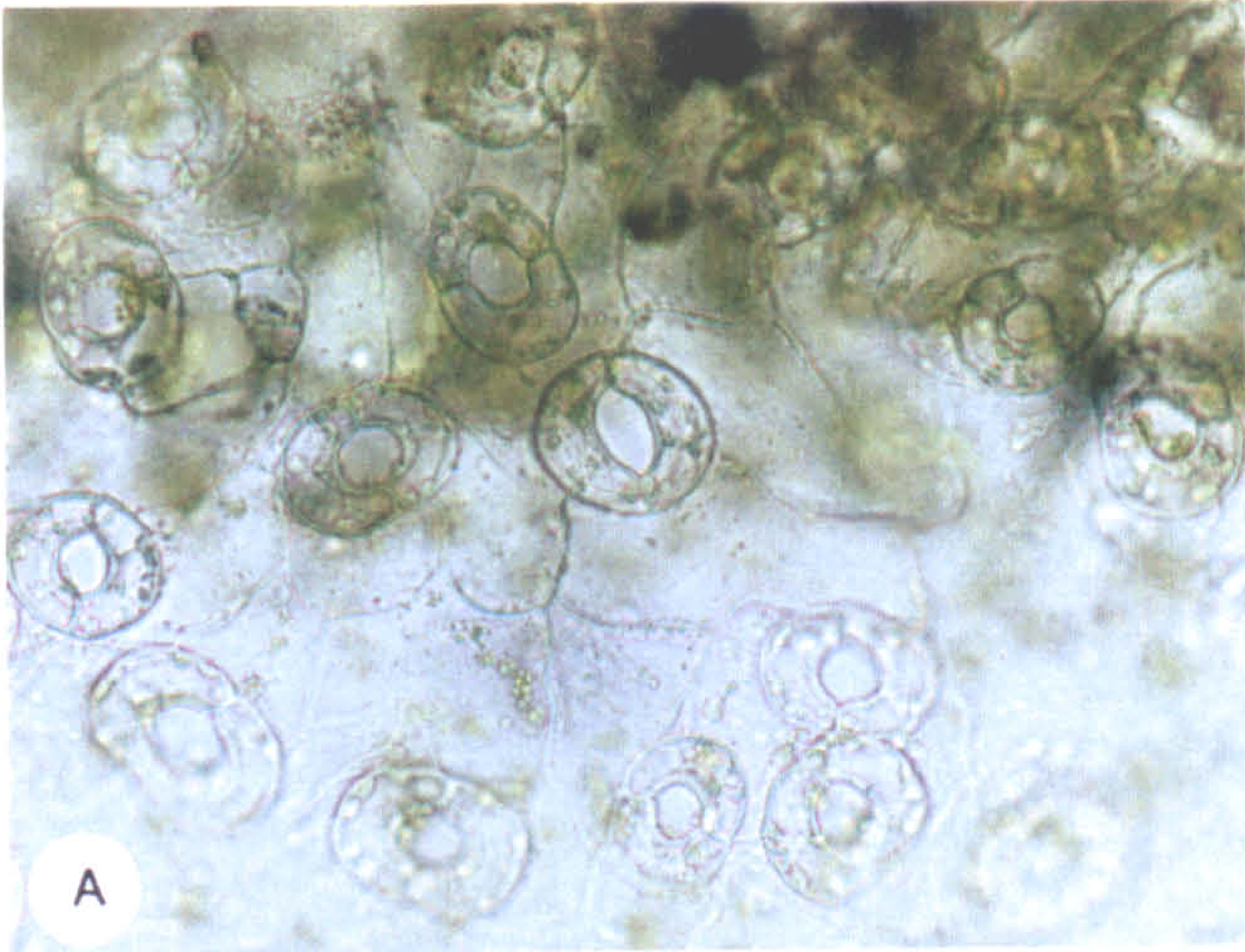
(A) sealed condition (silicone rubber bung); note that relatively larger stomata were densely arranged and remained gaping widely open.

(B) diffusive ventilation (polypropylene disc), note that large stomata were less densely arranged than in (A) and also remained widely open .

(C) fast forced ventilation (flow rate was 5 $cm^3$ $min^{-1}$); note smaller stomata and less densely arranged than in (A); stomata pores were narrower in width than in (A) and (B).

(D) very fast forced ventilation (flow rate was 10 $cm^3$ $min^{-1}$); note that smaller stomata were less densely arranged than in (A) and (B) and also stomatal pores were narrower in width than in (A), (B) and (C).

(E) *in vivo* (growth room conditions); note that few stomata which were smaller in size than in (A) - (D) and almost all closed.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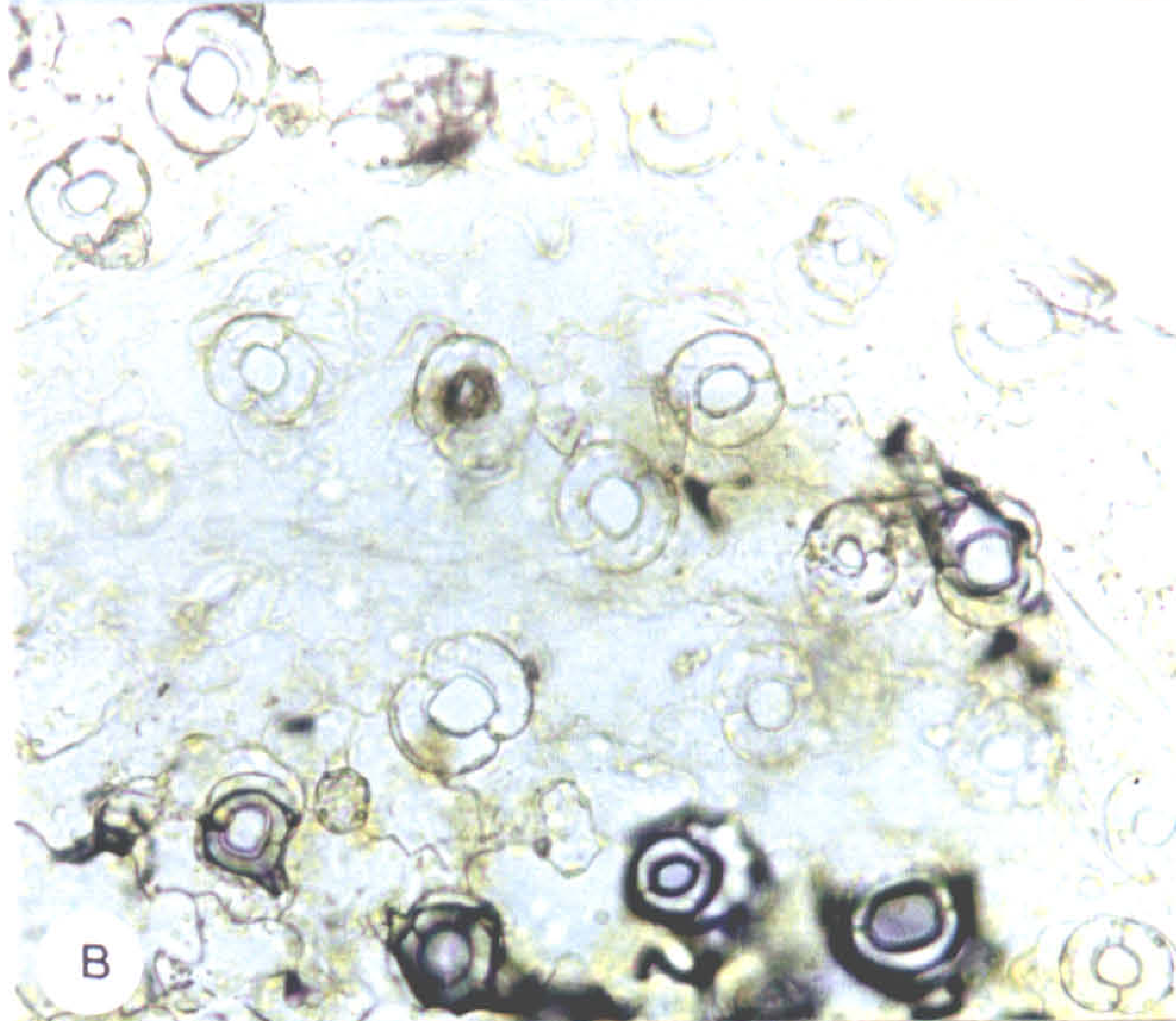
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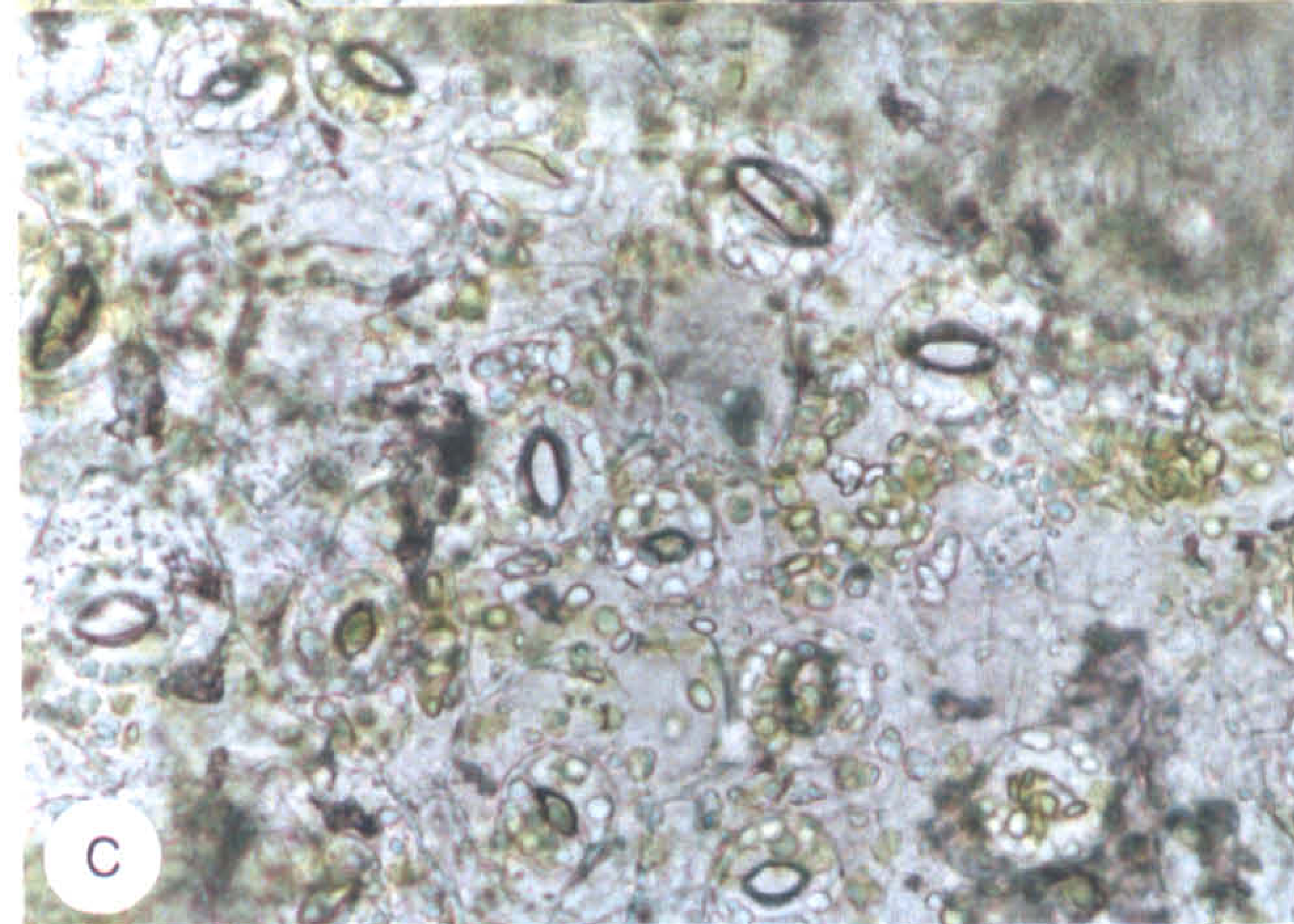
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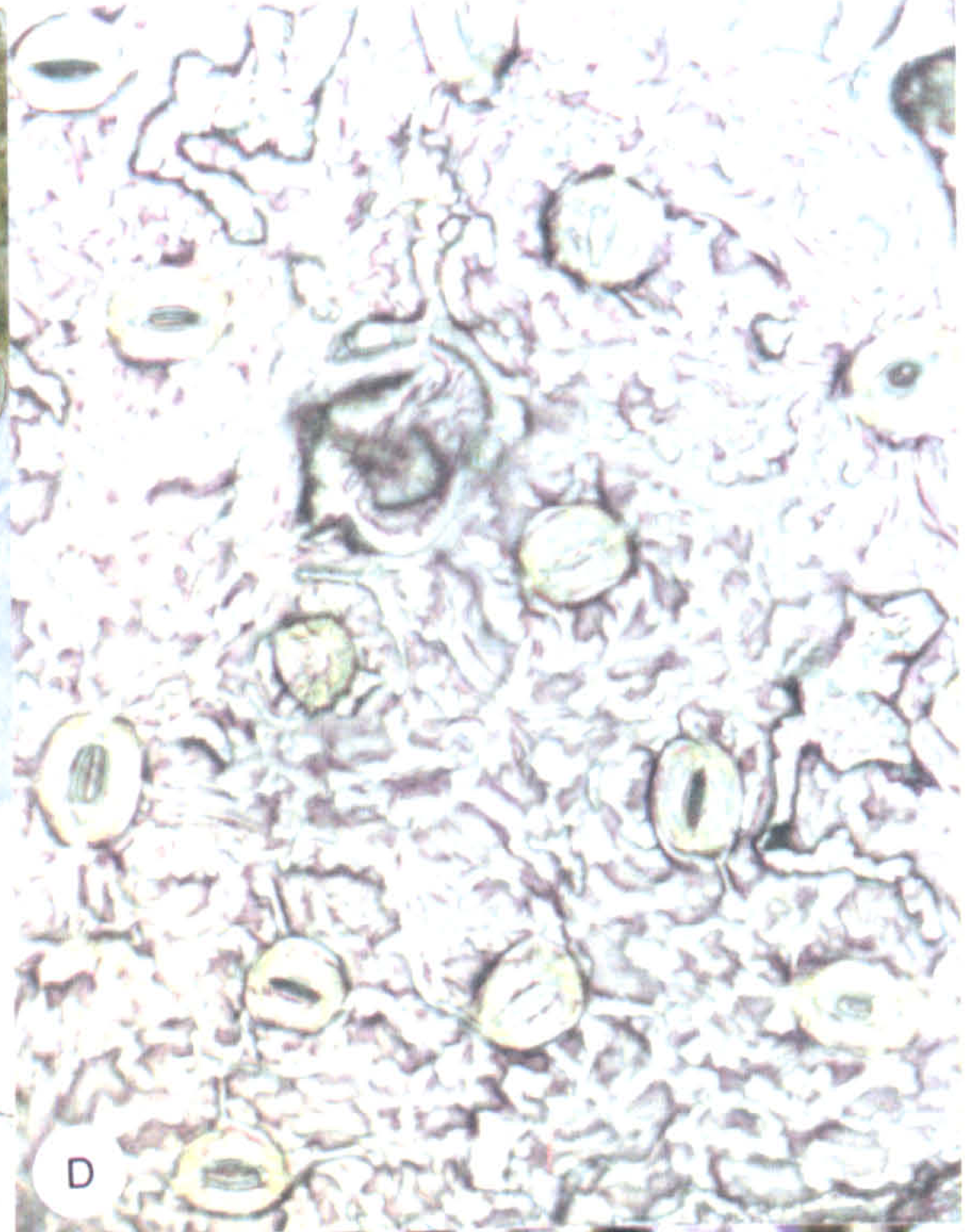
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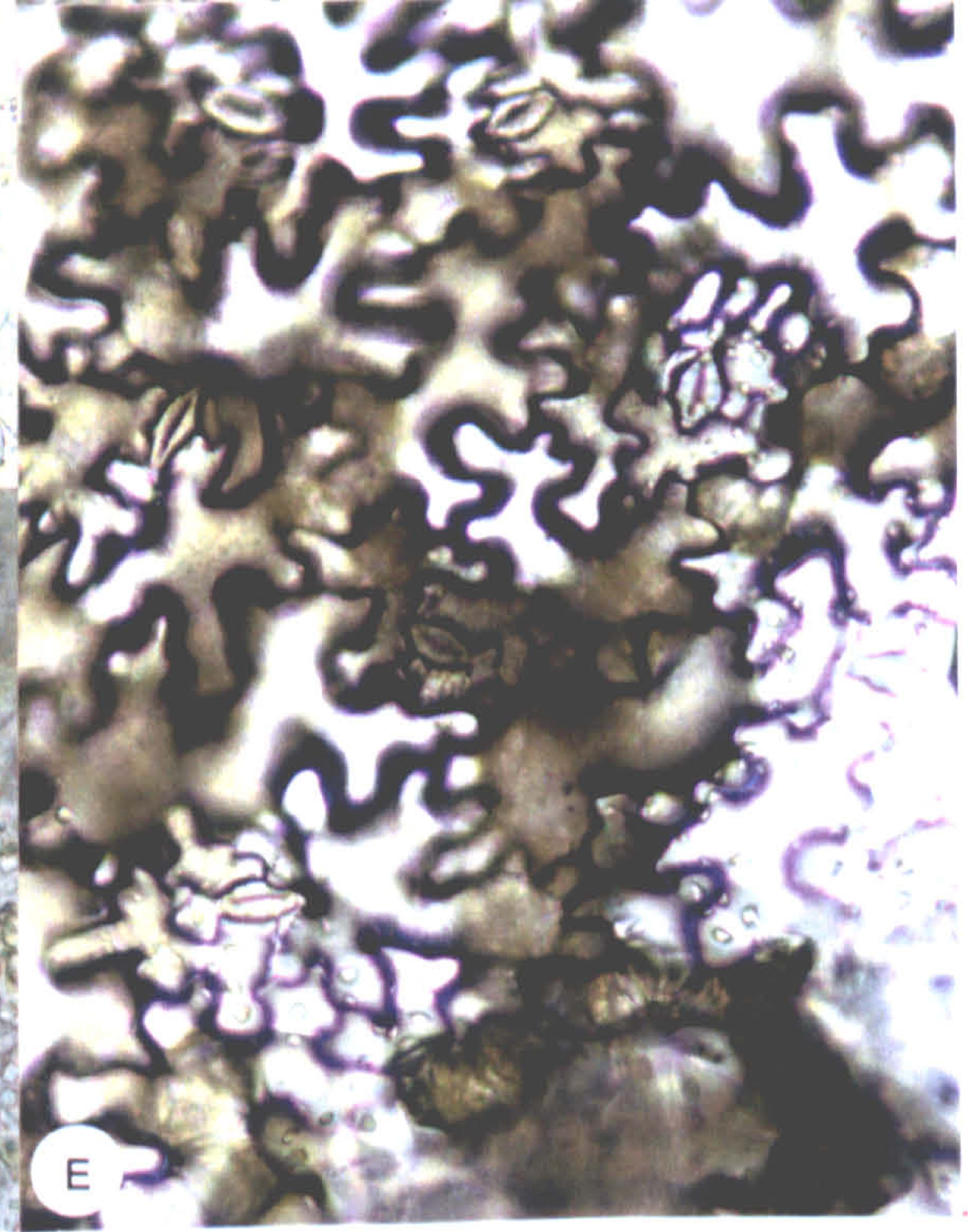
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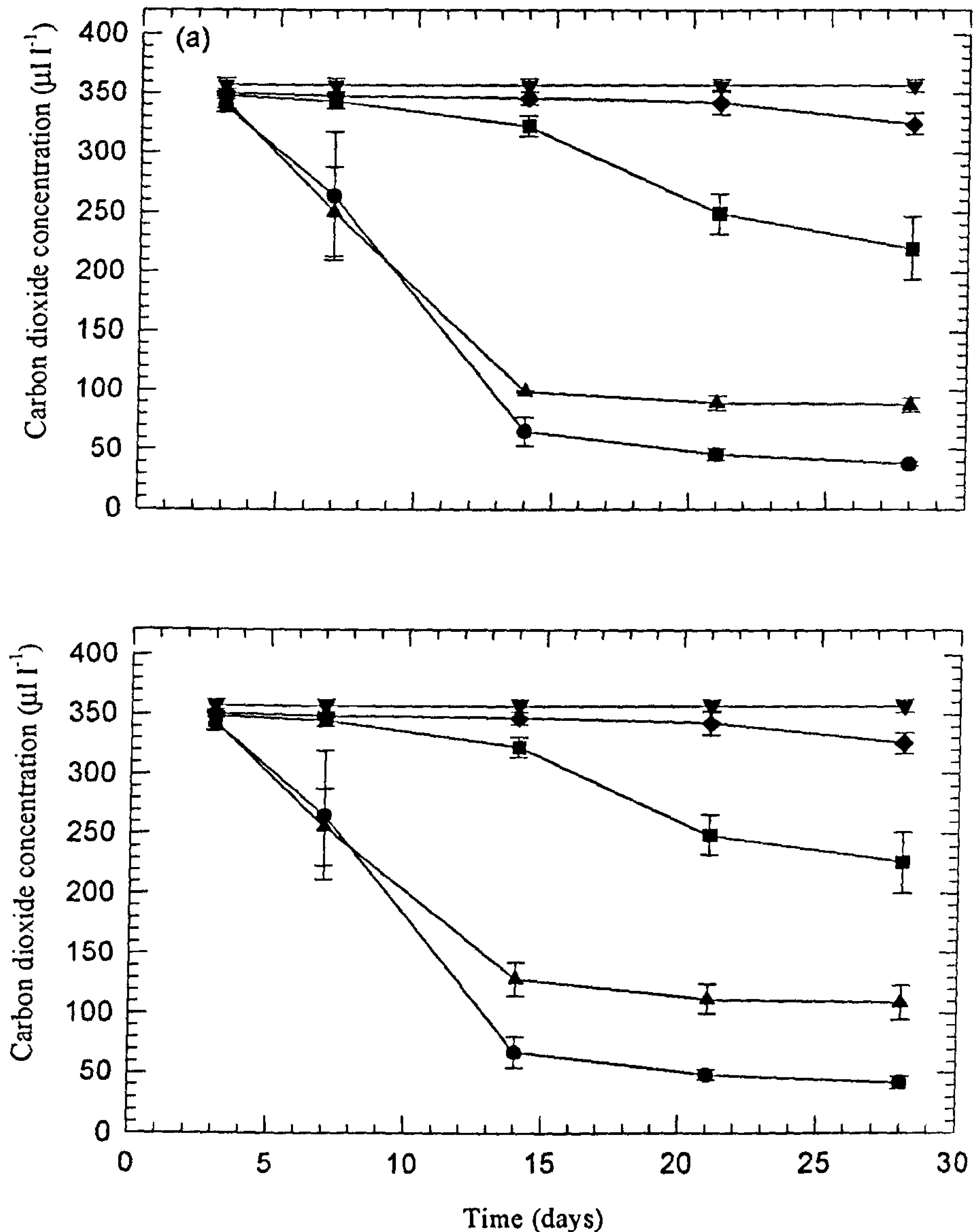



**Fig. 6.03.** Showing the $CO_2$ concentration in the culture head-space during the light period of (a) cauliflower plantlets and (b) tobacco plantlets grown *in vitro* under different types of ventilation; closed (each vessel was sealed with a silicone rubber bung ●), diffusive ventilation (each vessel was capped with a polypropylene disc Δ), fast forced ventilation (each vessel was fitted with a single ventilation apparatus) - flow rate was 5 $cm^3$ $min^{-1}$ ■, very fast forced ventilation (each vessel was fitted with two sets of ventilation apparatus) - flow rate was 10 $cm^3$ $min^{-1}$ ◆) and *in vivo* (growth room conditions▼). Cultures were grown at ca. 25°C with 8 hour dark and 16 hour light periods; PAR = 150 µmol $m^{-2}s^{-1}$. Volume of culture vessel = 60 $cm^3$. Each bar represents a mean ± SE of 5 replicates.

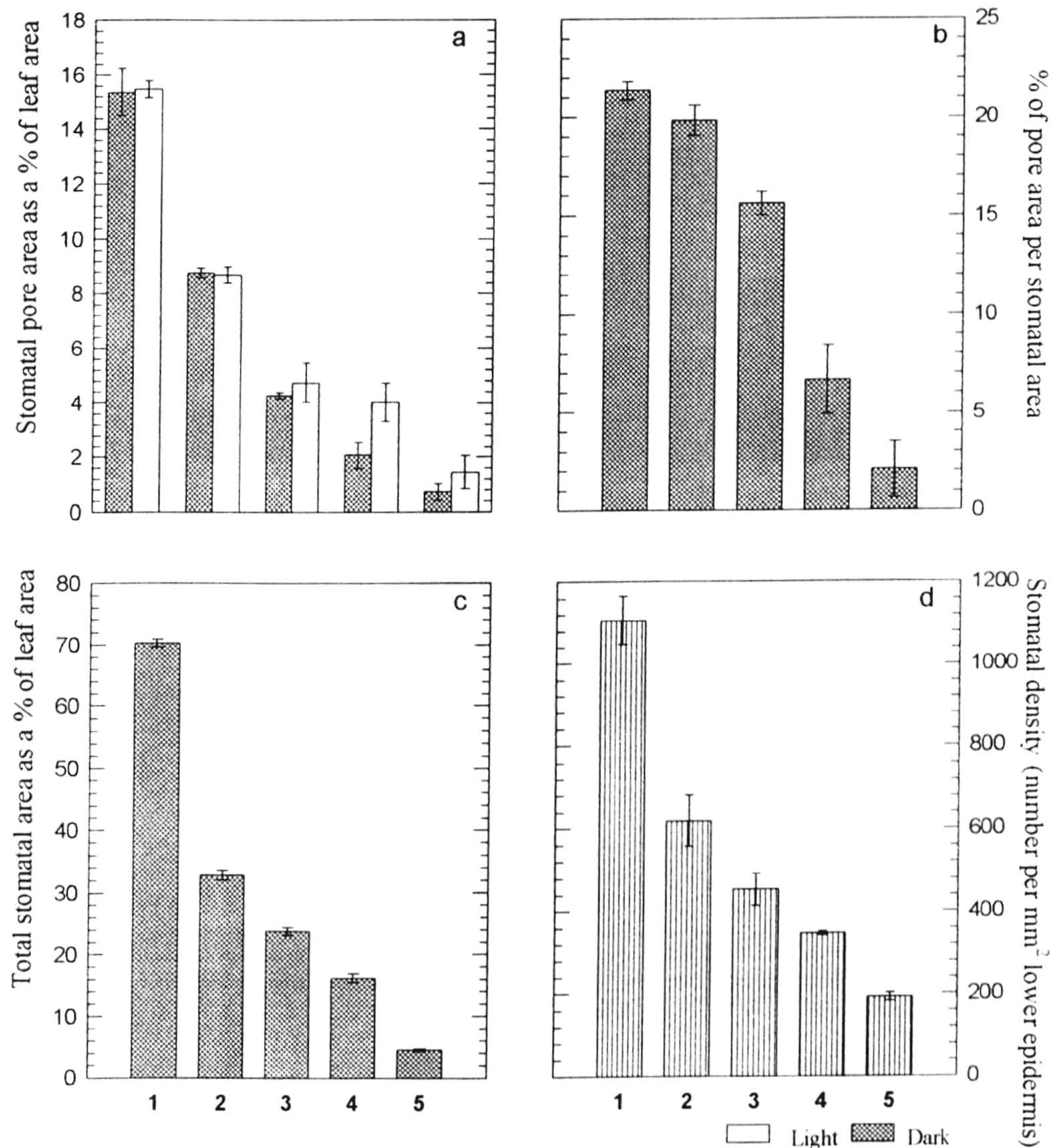



**Fig. 6.04.** Cauliflower : stomata of lower epidermis of 3rd or 4th leaf from apex : (a) stomatal pore area expressed as % of leaf area, (b) pore area per stomatal area (c) total stomatal area expressed as a % of leaf area and (d) stomatal density (numbers per $mm^2$ leaf area). 28 days old plantlets were grown under ① sealed condition (each vessel was sealed with a silicone rubber bung), ② diffusive ventilation (each vessel was capped with a polypropylene disc), ③ fast forced ventilation (each vessel was fitted with a single ventilation apparatus) - flow rate was 5 $cm^3$ $min^{-1}$ ④ very fast forced ventilation (each vessel was fitted with two sets of ventilation apparatus) - flow rate was 10 $cm^3$ $min^{-1}$ and ⑤ *in vivo* (growth room conditions). Volume of culture vessel = 60 $cm^3$. Cultures were grown at *ca.* 25°C with 8 hour dark and 16 hour light periods; PAR = 150 µmol $m^{-2}s^{-1}$. Each bar represents a mean ± SE of 3-4 samples (*ca.* 20 stomata each).

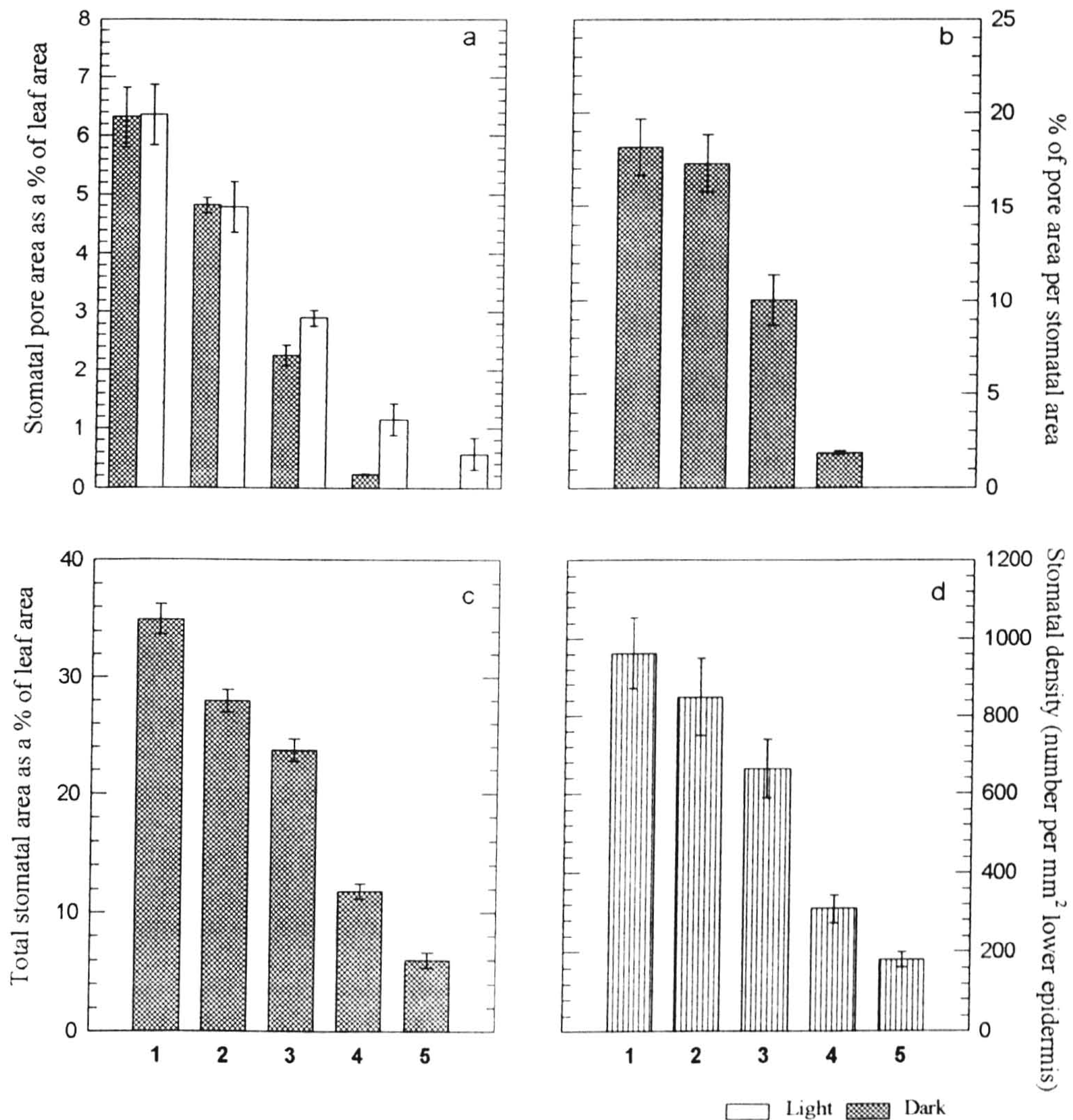



**Fig. 6.05.** Tobacco: stomata of lower epidermis of 3rd or 4th leaf from apex : (a) stomatal pore area expressed as % of leaf area, (b) pore area per stomatal area (c) total stomatal area expressed as a % of leaf area and (d) stomatal density (numbers per $mm^2$ leaf area). 28 days old plantlets were grown under ① sealed condition (each vessel was sealed with a silicone rubber bung), ② diffusive ventilation (each vessel was capped with a polypropylene disc), ③ fast forced ventilation (each vessel was fitted with a single ventilation apparatus) - flow rate was 5 $cm^3$ $min^{-1}$ ④ very fast forced ventilation (each vessel was fitted with two sets of ventilation apparatus) - flow rate was 10 $cm^3$ $min^{-1}$ and ⑤ *in vivo* (growth room conditions). Volume of culture vessel = 60 $cm^3$. Cultures were grown at *ca.* 25°C with 8 hour dark and 16 hour light periods; PAR = 150 µmol $m^{-2}s^{-1}$. Each bar represents a mean ± SE of 3-4 samples (*ca.* 20 stomata each).

treatment for cauliflower and tobacco respectively. For the *in vivo* treatments the corresponding values were even lower at 0.18X and 0.21X respectively.

These findings are in accordance with the results of Wetzstein and Sommer (1983) where stomatal densities of *Liquidambar styraciflua* were shown to be significantly greater in cultured than in field-grown or acclimated plantlets. Sciutti and Morin (1993) also reported that stomatal density could be markedly increased in leaves of *in vitro* grown plum plantlets by increasing the relative humidity in the culture atmosphere. In this study, also, the highest stomatal densities were associated with the treatments with the highest RH, and *vice versa* (Figs. 6.04, 6.05).

The concentration of carbon dioxide may also have played a major role in influencing stomatal density in leaves of both cauliflower and tobacco plantlets. As described in Figure 6.03, for both species the $CO_2$ concentrations were significantly lower in both sealed and diffusive treatments where the highest density of stomata were noticed. Also in fast flow and very fast flow ventilation treatments lower densities of stomata were associated with higher $CO_2$ concentrations. This would accord with the findings of Woodward and Kelly (1995), who investigated a hundred species and found reductions in stomatal density (*ca.* 14%) in 74% of the species. The $CO_2$ enrichment was 350- 700 $\mu$mol mol$^{-1}$ and the effects were especially marked in the members of Hamamelidae and Rosidae .

The ethylene concentration in the culture vessels is another factor that may have affected stomatal densities. In the present study the accumulation of ethylene was noted in culture vessels subjected to sealed and the diffusive ventilation (Fig. 6.02) where the highest stomatal densities were recorded, and *vice versa.*

#### 6.3.2.2. Stomatal size

Note: Stomatal length refers to the distance between the ends of the guard cells, and the width is the distance transversely "across" them.

In general stomatal sizes appeared to be inversely related to efficiencies of ventilation (Figs 6.06, 6.07). For example, in the sealed condition in darkness, in both cauliflower and tobacco, the average length of each stoma was 31 and 13.6 µm respectively; these were 1.9X and 1.7X greater than those from the *in vivo* treatment. The most significant differences were observed in stomatal widths which were, in the dark period, for cauliflower and tobacco respectively, 2.3X and *ca* 3.0X the values for the *in vivo* condition. It should also be mentioned that in the sealed and to some extent in the diffusive treatments in some parts of the leaves some of the stomata were narrow in width and resembled those from the better ventilated treatments. These might have been immature stomata which had not yet opened (Plates 6.03a, 6.04a, 6.05a, 6.11). Also, in diffusive ventilation, the very young, green, immature leaves of tobacco were found to have this type of stoma.

### 6.3.2.3. Stomatal area

Relatively larger areas were occupied by stomata in both the closed and diffusive systems in both the species (Figs. 6.04c, 6.05c). In the dark, in cauliflower and tobacco respectively in the sealed treatment, 70% and 34.5% of the leaf areas were occupied by stomata compared to 33% and 28% in diffusive treatments. The stomatal area was significantly lower in *in vivo* grown plantlets and also in very fast flow ventilation treatment (Figs. 6.04c, 6.05c; Plates 6.01, 6.02). These effects were no doubt due to the lower stomatal density and the smaller size of the stomata found in the latter two types of treatment (described above). The fast flow ventilation treatment exhibited a higher stomatal area than those of the very fast flow ventilation and *in vivo* treatments, but very much lower than the sealed or diffusive ones.

### 6.3.2.4. Stomatal opening and pore sizes

In both species, one very noticeable feature between treatments was that in the sealed and diffusive treatments a large proportion of the stomata seemed to be gaping wide open, so that the width of the stoma and of the pore were greater than the corresponding

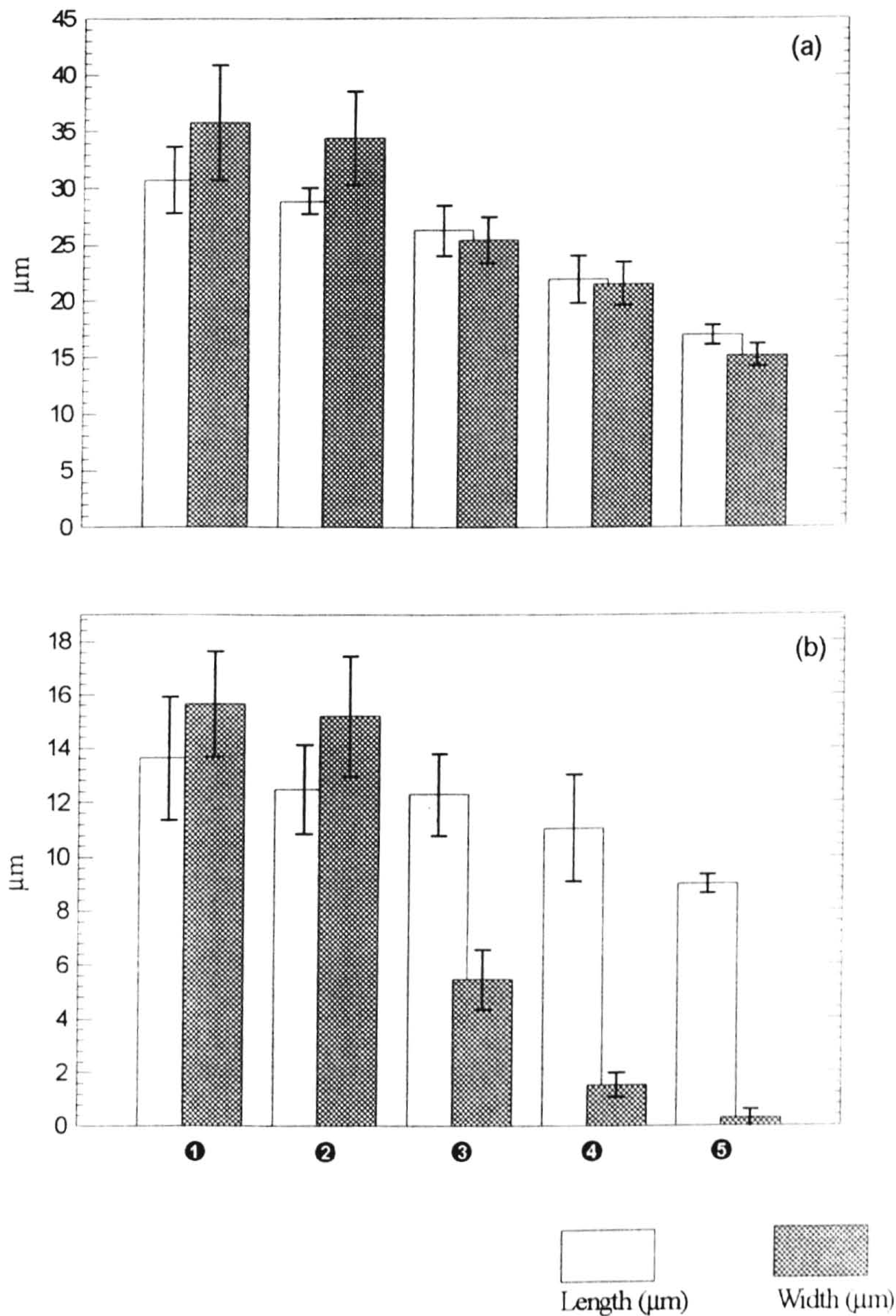



**Fig. 6.06.** Cauliflower : stomata of lower epidermis of 3rd or 4th leaf from apex : (a) length and width (µm) of stomata and (b) length and width (µm) of stomatal opening. 28 days old plantlets were grown under ❶ sealed condition (each vessel was sealed with a silicone rubber bung), ❷ diffusive ventilation (each vessel was capped with a polypropylene disc), ❸ fast forced ventilation (each vessel was fitted with a single ventilation apparatus) - flow rate was 5 $cm^3$ $min^{-1}$ ❹ very fast forced ventilation (each vessel was fitted with two sets of ventilation apparatus) - flow rate was 10 $cm^3$ $min^{-1}$ and ❺ *in vivo* (growth room conditions). Volume of culture vessel = 60 $cm^3$. Cultures were grown at *ca.* 25°C with 8 hour dark and 16 hour light periods; PAR = 150 µmol $m^{-2}s^{-1}$. Each bar represents a mean ± SE of 20 replicates.

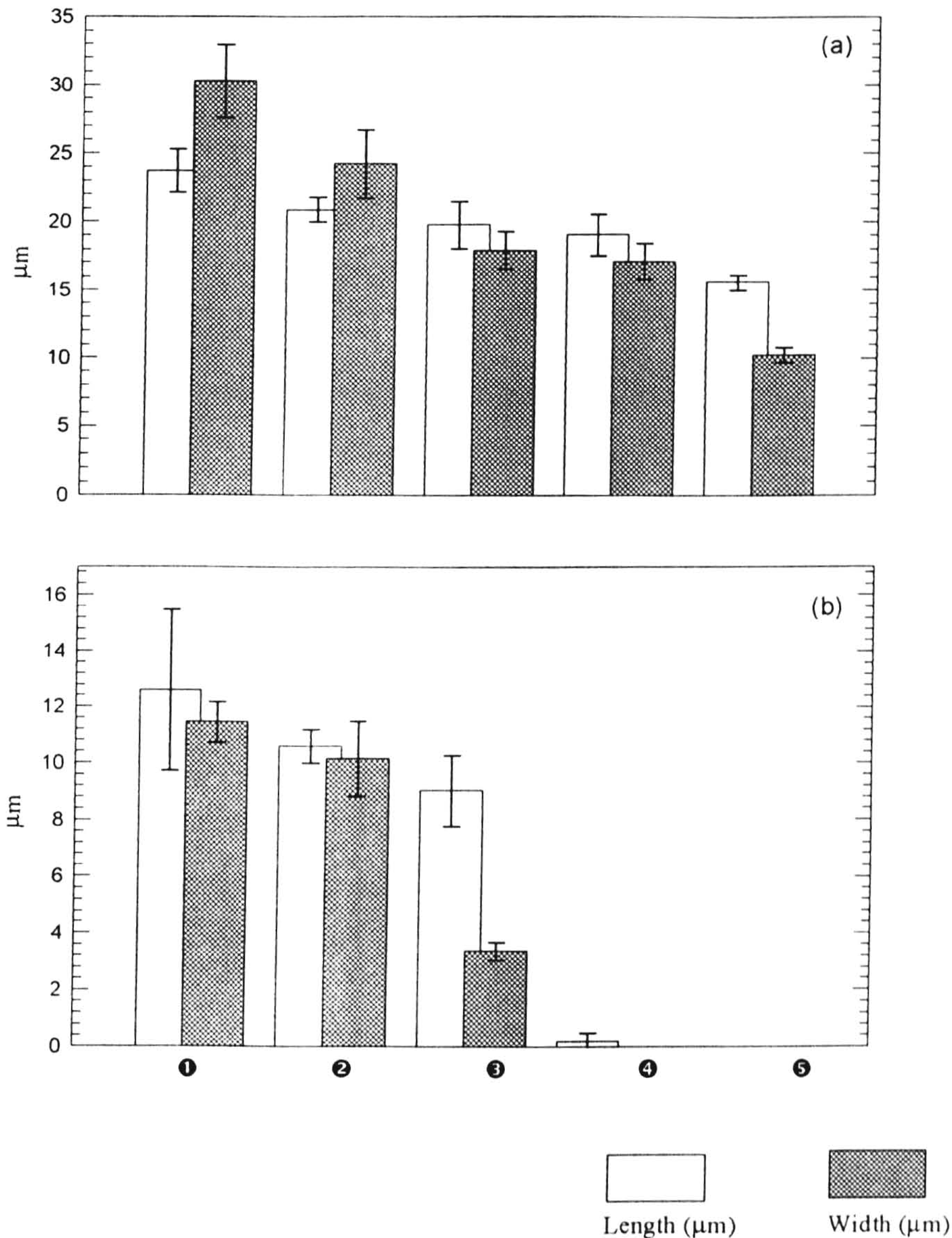



**Fig. 6.07.** Tobacco : stomata of lower epidermis of 3rd or 4th leaf from apex : (a) length and width (µm) of stomata and (b) length and width (µm) of stomatal opening. 28 days old plantlets were grown under ❶ sealed condition (each vessel was sealed with a silicone rubber bung), ❷ diffusive ventilation (each vessel was capped with a polypropylene disc), ❸ fast forced ventilation (each vessel was fitted with a single ventilation apparatus) - flow rate was 5 $cm^3$ $min^{-1}$ ❹ very fast forced ventilation (each vessel was fitted with two sets of ventilation apparatus) - flow rate was 10 $cm^3$ $min^{-1}$ and ❺ *in vivo* (growth room conditions). Volume of culture vessel = 60 $cm^3$. Cultures were grown at *ca.* 25°C with 8 hour dark and 16 hour light periods; PAR = 150 µmol $m^{-2}s^{-1}$. Each bar represents a mean ± SE of 20 replicates.

## PLATE : 6.03

**Cauliflower :** Stomata of lower epidermis of 3rd or 4th leaf from apex from shoot cuttings after 28 days and from light period. Culture vessel volume was 60 $cm^3$. Growth room conditions: *ca.* 25°C with 8 hours dark and 16 hours light periods; PAR = 150 $\mu mol\ m^{-2}s^{-1}$; RH = 26 - 32%. Plantlets were grown under different types of ventilation and also *in vivo* condition as indicated bellow (X275).

Slivers of lower epidermis were stained in 0.02% aqueous auramine and photographed under blue light to show waxes fluorescing yellow (also chlorophyll shown fluorescing red).

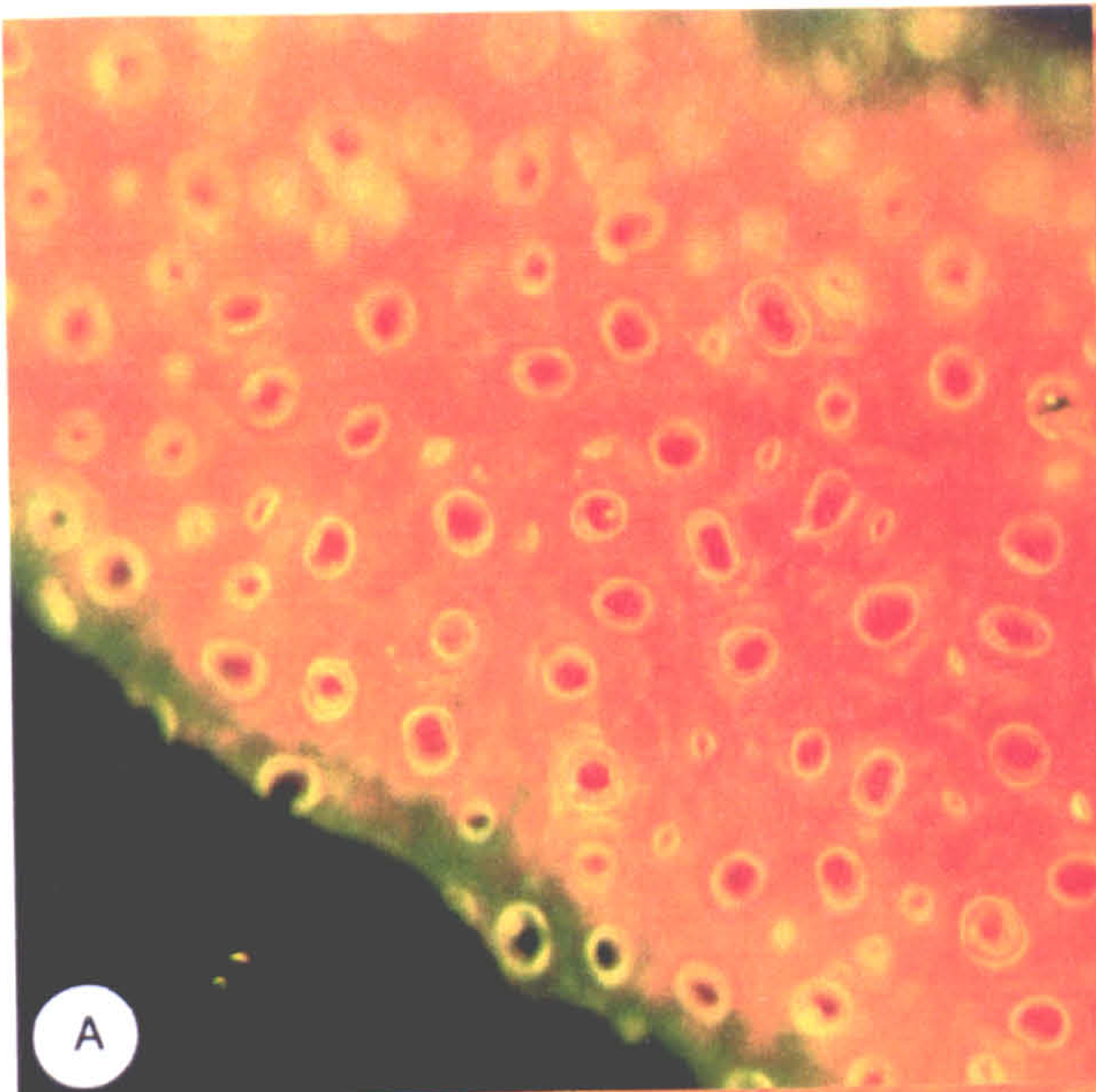
(A) sealed condition(silicone rubber bung); note that relatively larger stomata, were densely arranged and gaping widely open. Some stomata, perhaps immature were smaller and appeared partially open or closed.

(B) diffusive ventilation (polypropylene disc); note that large stomata were slightly less dense than in (A) and also gaping widely open. Note apparently immature stomata as in (A).

(C) fast forced ventilation (flow rate was 5 $cm^3\ min^{-1}$); note that relatively smaller stomata were less densely arranged than in (A) and (B) and stomatal pores were generally narrower in width.

(D) very fast forced ventilation (flow rate was 10 $cm^3\ min^{-1}$ ); note that stomata were smaller less dense than in (A) and (B) and also stomatal pores were generally narrower in width.

(E) *in vivo* (growth room conditions); note that stomata were far less dense and with considerably narrower stomatal pores than in (A) - (D).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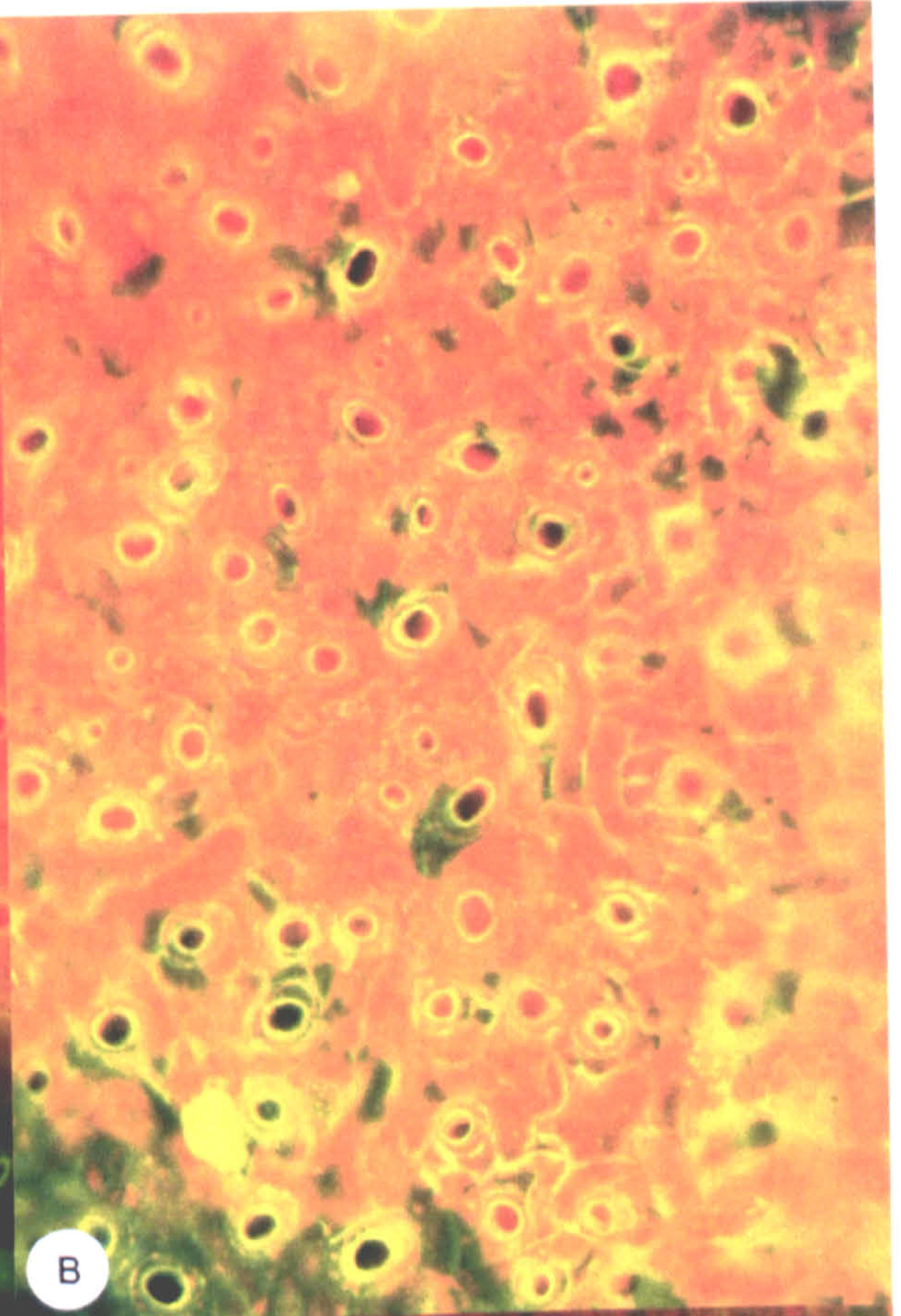
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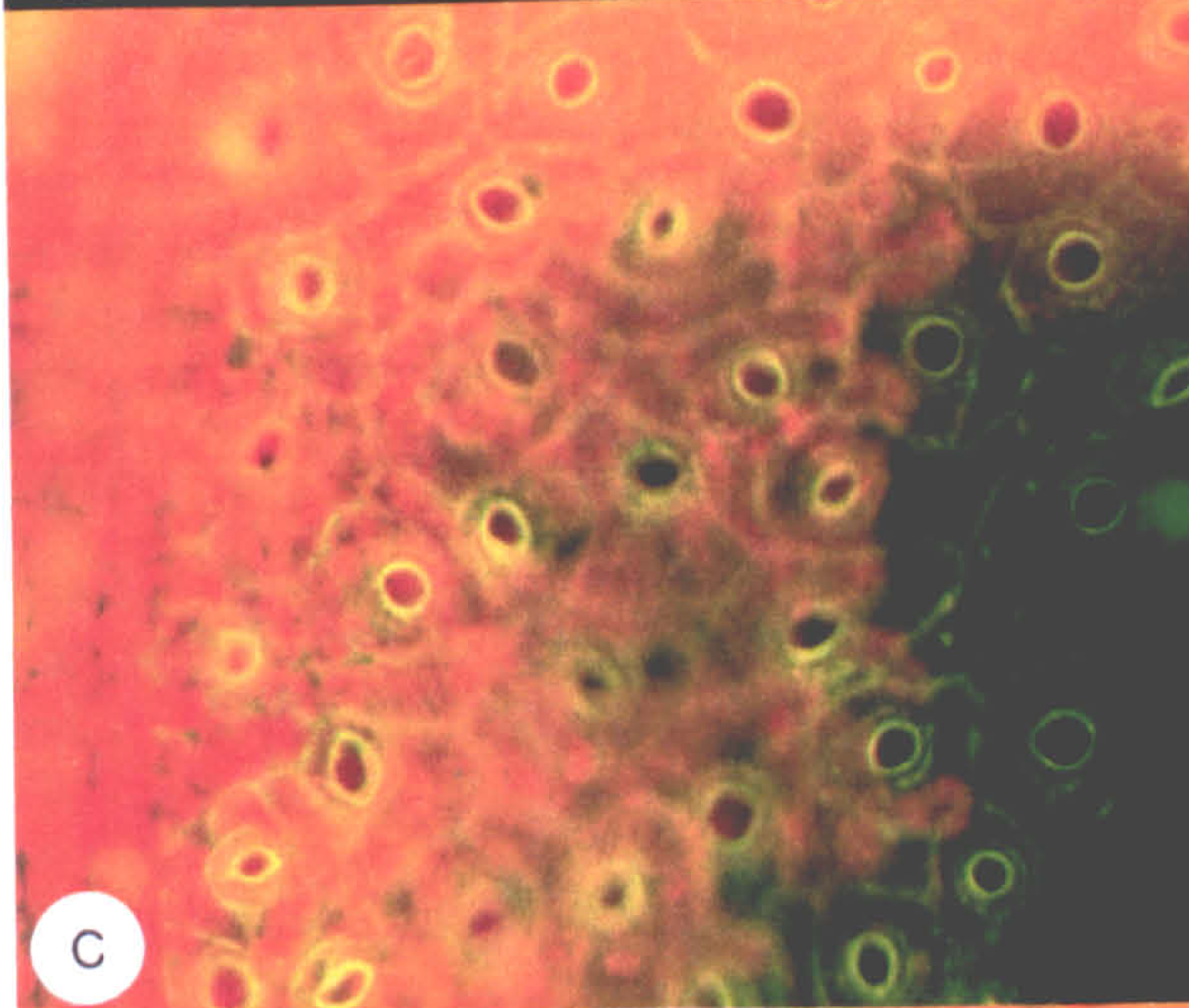
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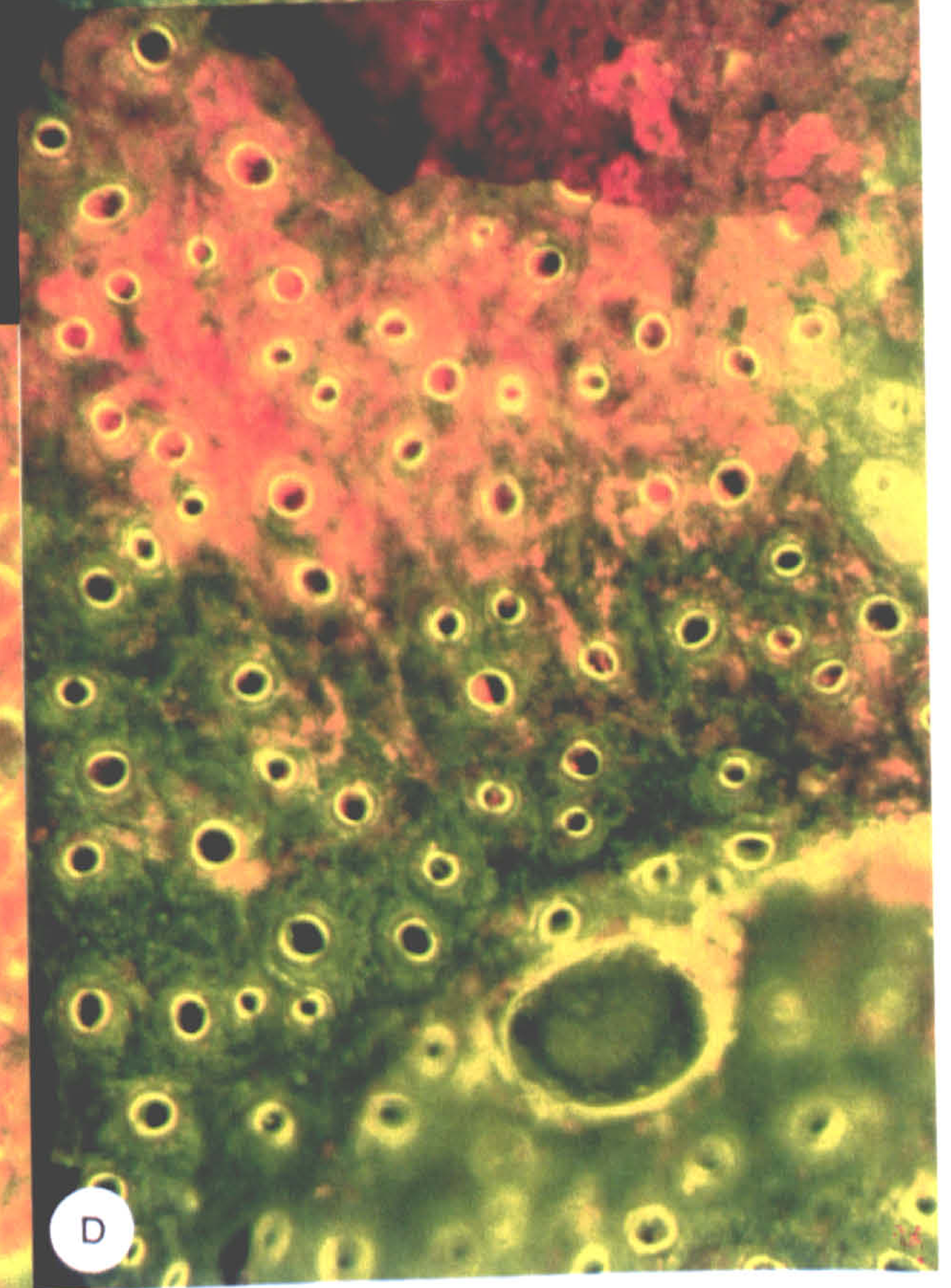
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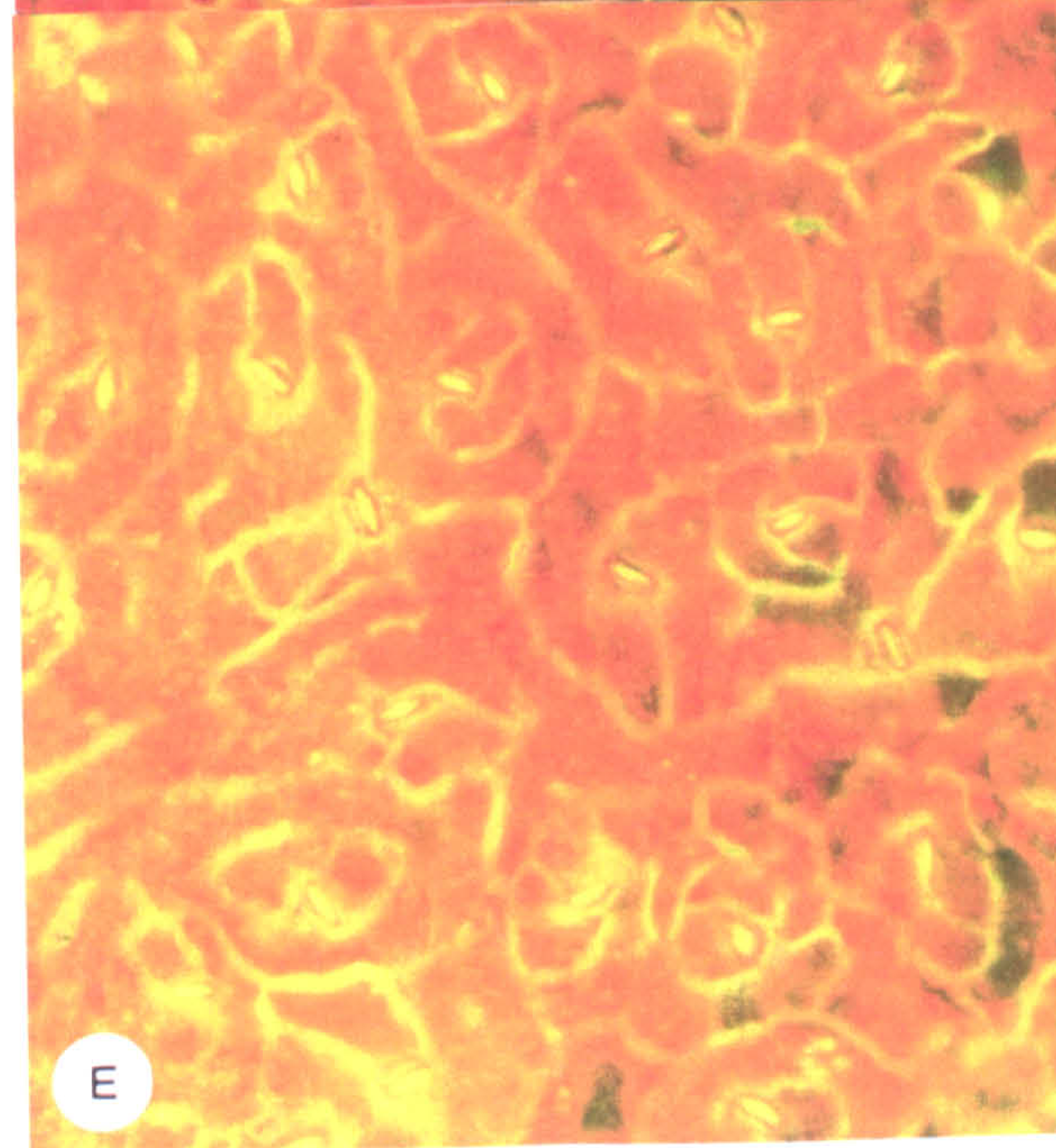
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**PLATE : 6.04**

**Cauliflower :** Stomata of lower epidermis of 3rd or 4th leaf from apex from shoot cuttings after 28 days and from light period. Culture vessel volume was 60 $cm^3$. Growth room conditions: *ca.* 25°C with 8 hours dark and 16 hours light periods; PAR = 150 $\mu mol\ m^{-2}s^{-1}$; RH = 26 - 32%. Plantlets were grown under different types of ventilation and also *in vivo* condition as indicated bellow (X688).

Slivers of lower epidermis were stained in 0.02% aqueous auramine and photographed under blue light to show waxes fluorescing yellow (also chlorophyll shown fluorescing red).

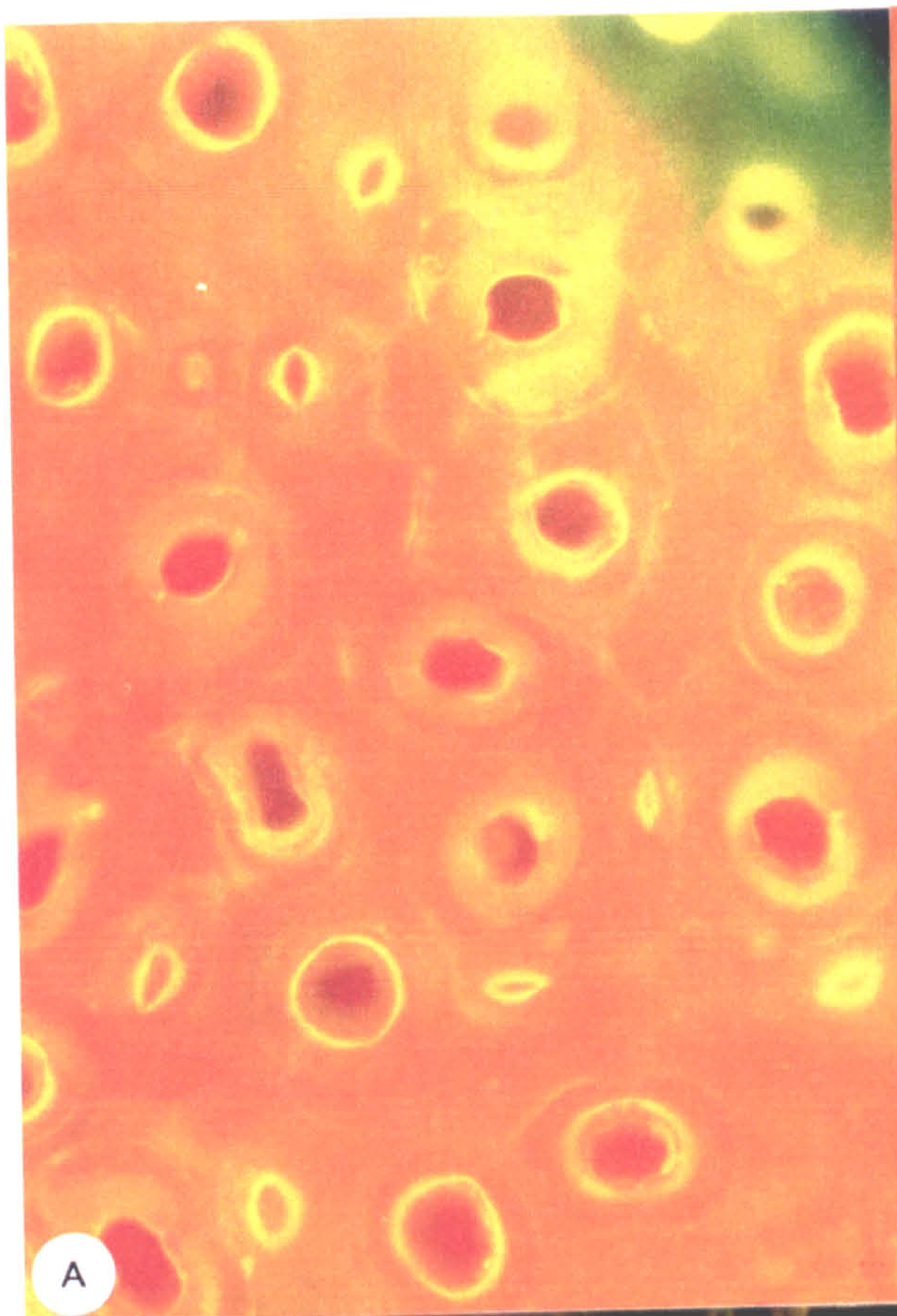
(A) sealed condition (silicone rubber bung); note that relatively larger stomata, were densely arranged and gaping widely open. Some stomata, perhaps immature were smaller and appeared partially open or closed.

(B) diffusive ventilation (polypropylene disc), note that large stomata were slightly less dense than in (A) and also gaping widely open; stomatal waxes were less apparent than in the other treatments. Note apparently immature stomata as in (A).

(C) fast forced ventilation (flow rate was 5 $cm^3\ min^{-1}$); note that relatively smaller stomata were less densely arranged than in (A) and (B) and stomatal pores were generally narrower in width.

(D) very fast forced ventilation (flow rate was 10 $cm^3\ min^{-1}$ ); note that stomata were smaller less dense than in (A) and (B) and also stomatal pores were generally narrower in width. Epicuticular waxes are fluorescing yellow (➔)

(E) *in vivo* (growth room conditions); note that stomata were far less dense, smaller and with considerably narrower stomatal pores than in (A) - (D).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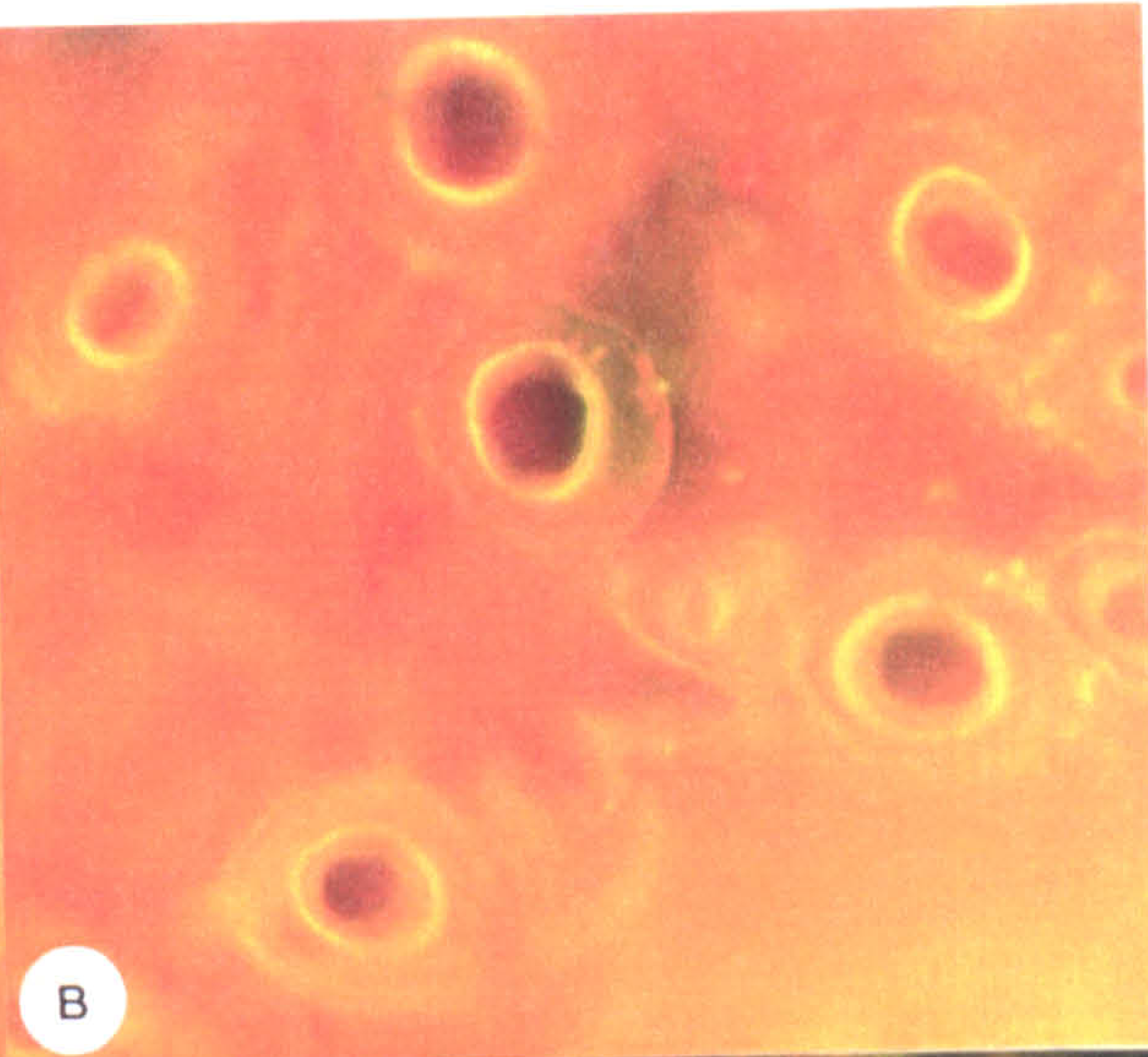
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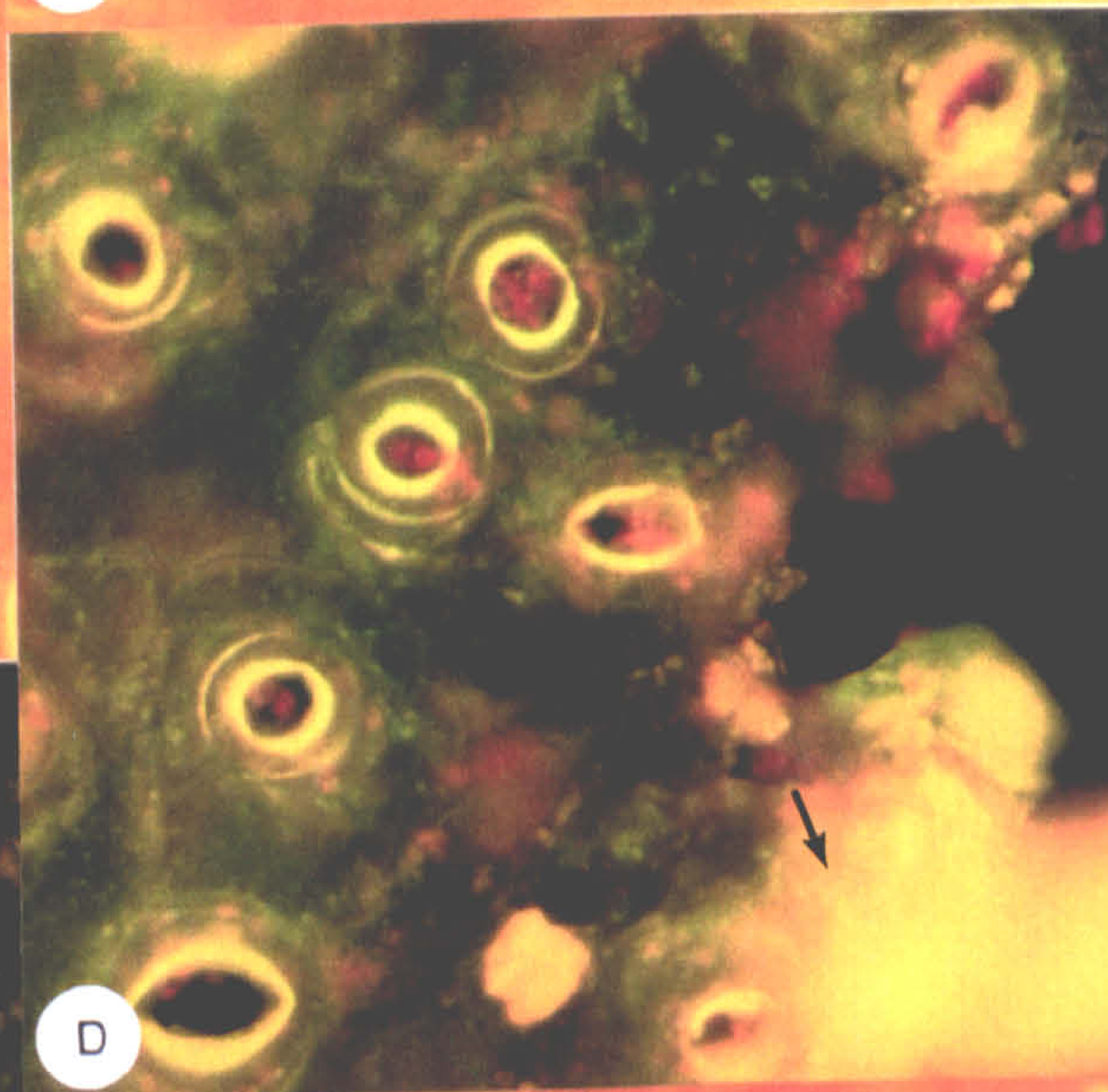
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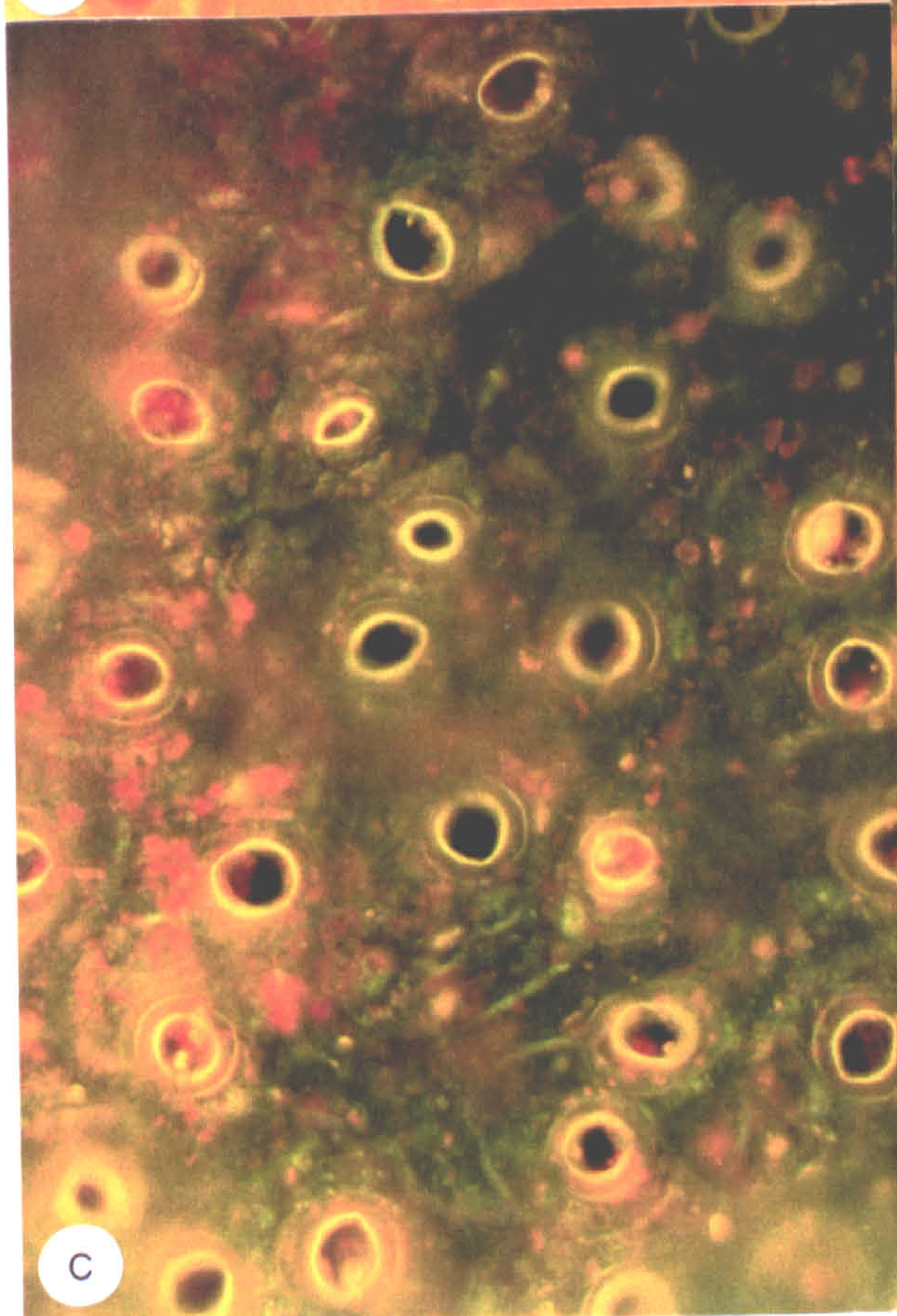
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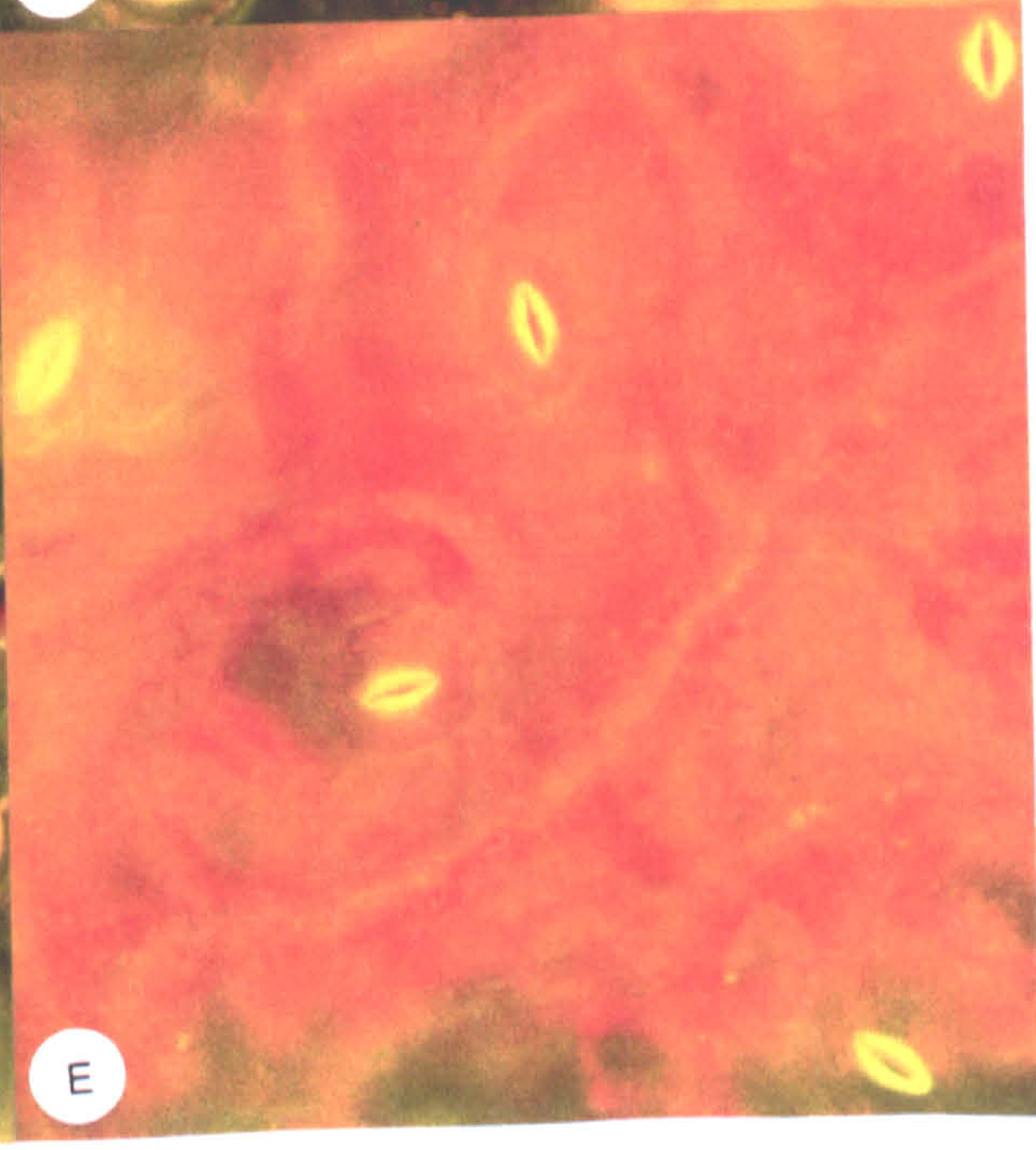
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## PLATE : 6.05

**Tobacco :** Stomata of lower epidermis of 3rd or 4th leaf from apex from shoot cuttings after 28 days and from light period. Culture vessel volume was 60 $cm^3$. Growth room conditions: *ca.* 25ºC with 8 hours dark and 16 hours light periods; PAR = 150 µmol $m^{-2}s^{-1}$; RH = 26 - 32%. Plantlets were grown under different types of ventilation and also *in vivo* condition as indicated bellow (X275).

Small pieces of leaf were stained in 0.02% aqueous auramine and the lower epidermis photographed under blue light to show waxes fluorescing yellow (also chlorophyll shown fluorescing red).

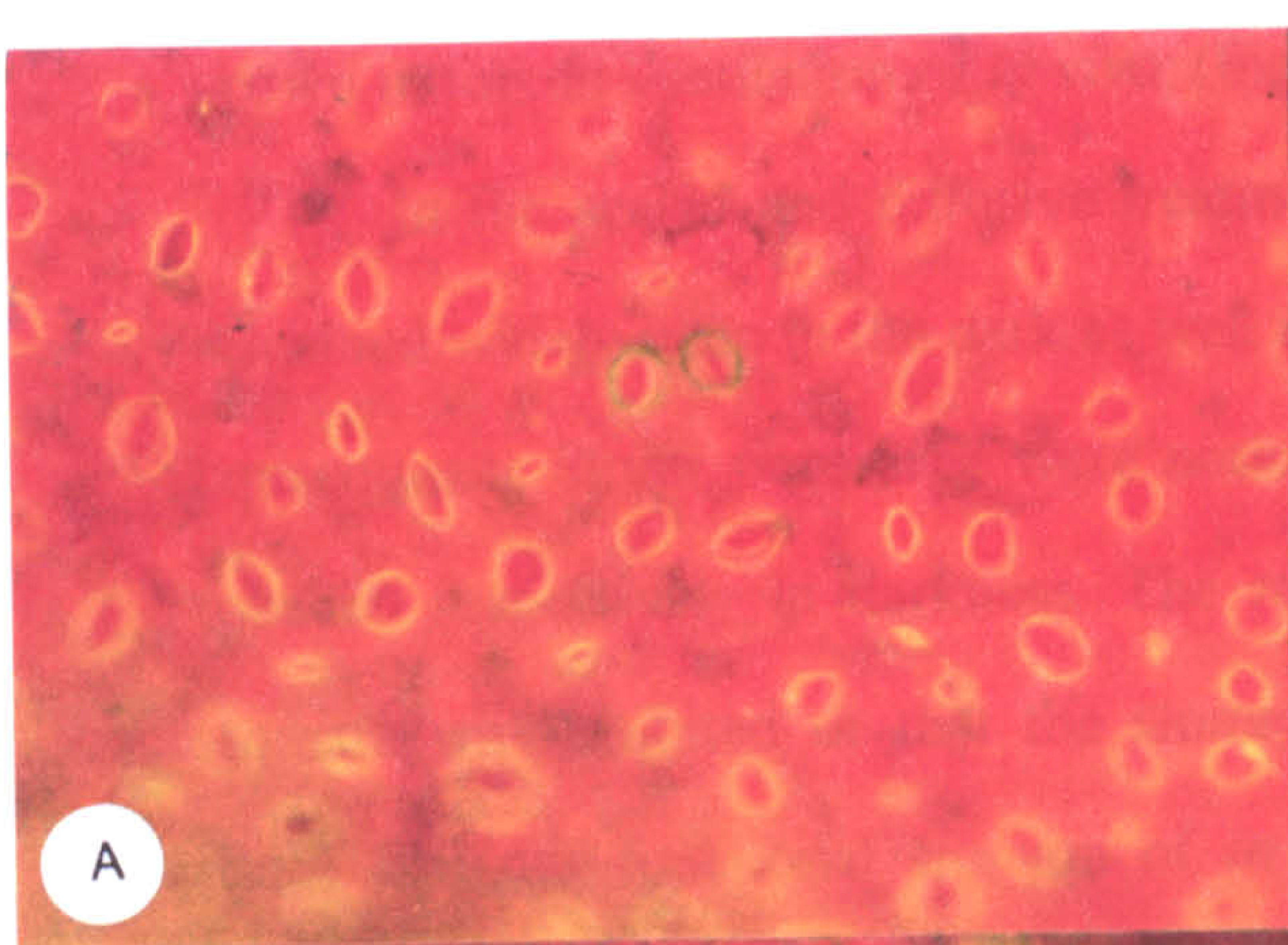
(A) sealed condition (silicone rubber bung); note that relatively larger stomata, were densely arranged and widely open. Some stomata, perhaps immature were smaller and appeared partially open or closed.

(B) diffusive ventilation (polypropylene disc), note large open stomata; densities varied and were some times as high as (A).

(C) fast forced ventilation (flow rate = 5 $cm^3$ $min^{-1}$); note stomata were more closed than in (A) and (B) and less densely arranged than in (A). Epicuticular waxes are fluorescing yellow (➔)

(D) very fast forced ventilation (flow rate = 10 $cm^3$ $min^{-1}$ ); note that stomata were similar to those in (C) but generally more closed.

(E) *in vivo* (growth room conditions); note that few stomata which were smaller in size with very narrow stomatal pores. Epicuticular waxes are fluorescing yellow (➔) epidermal hair (h) also has waxy wall.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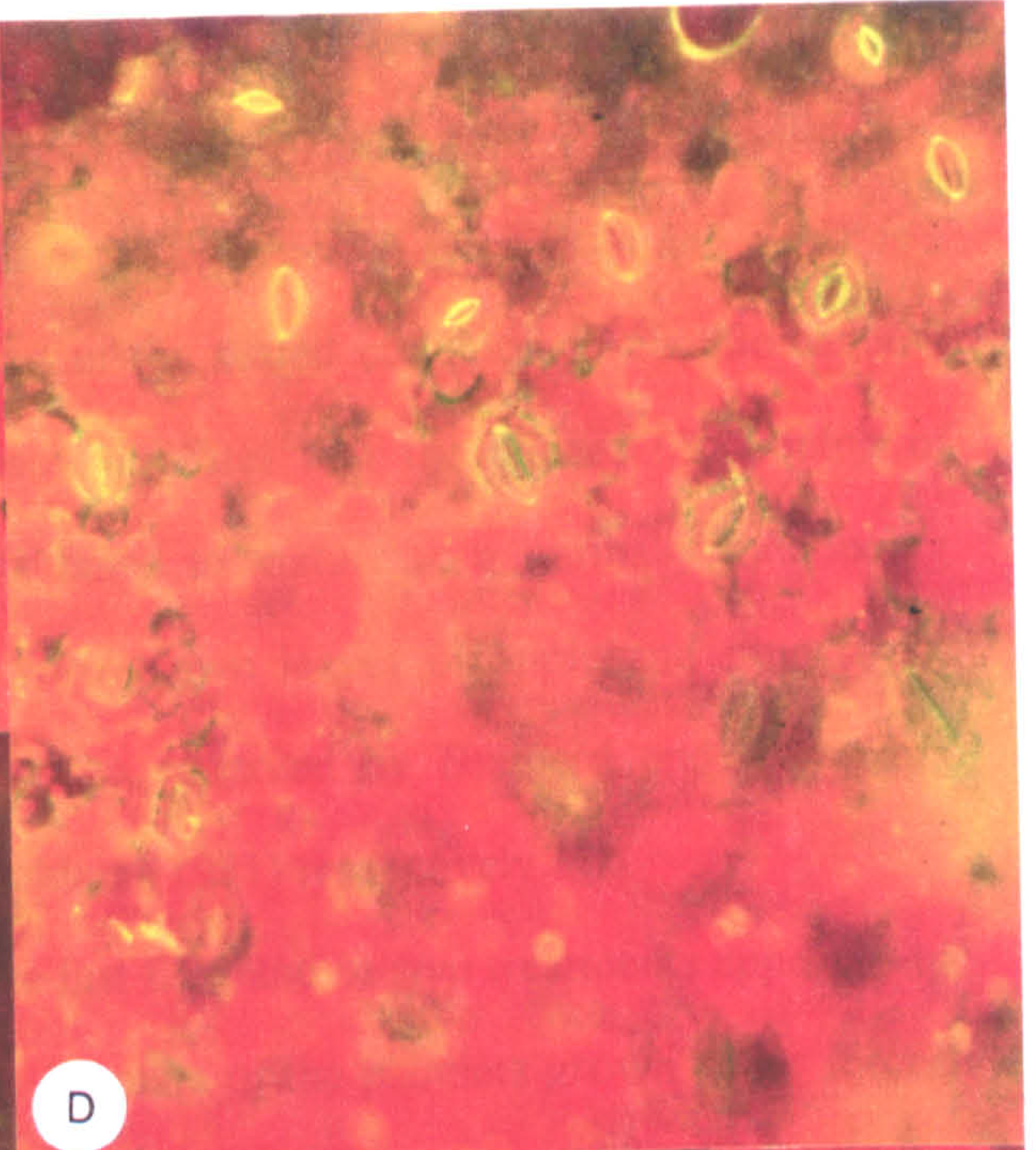
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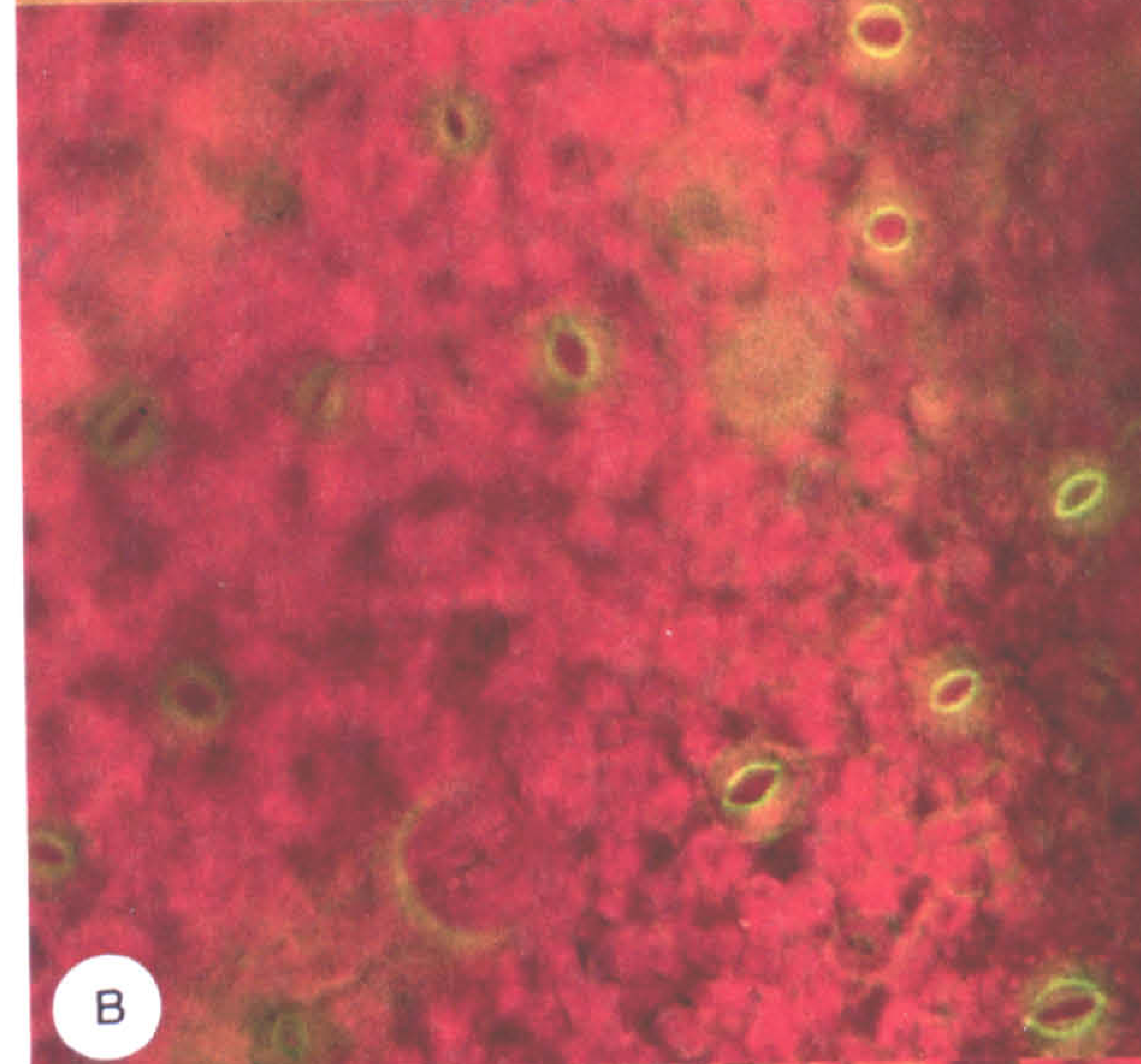
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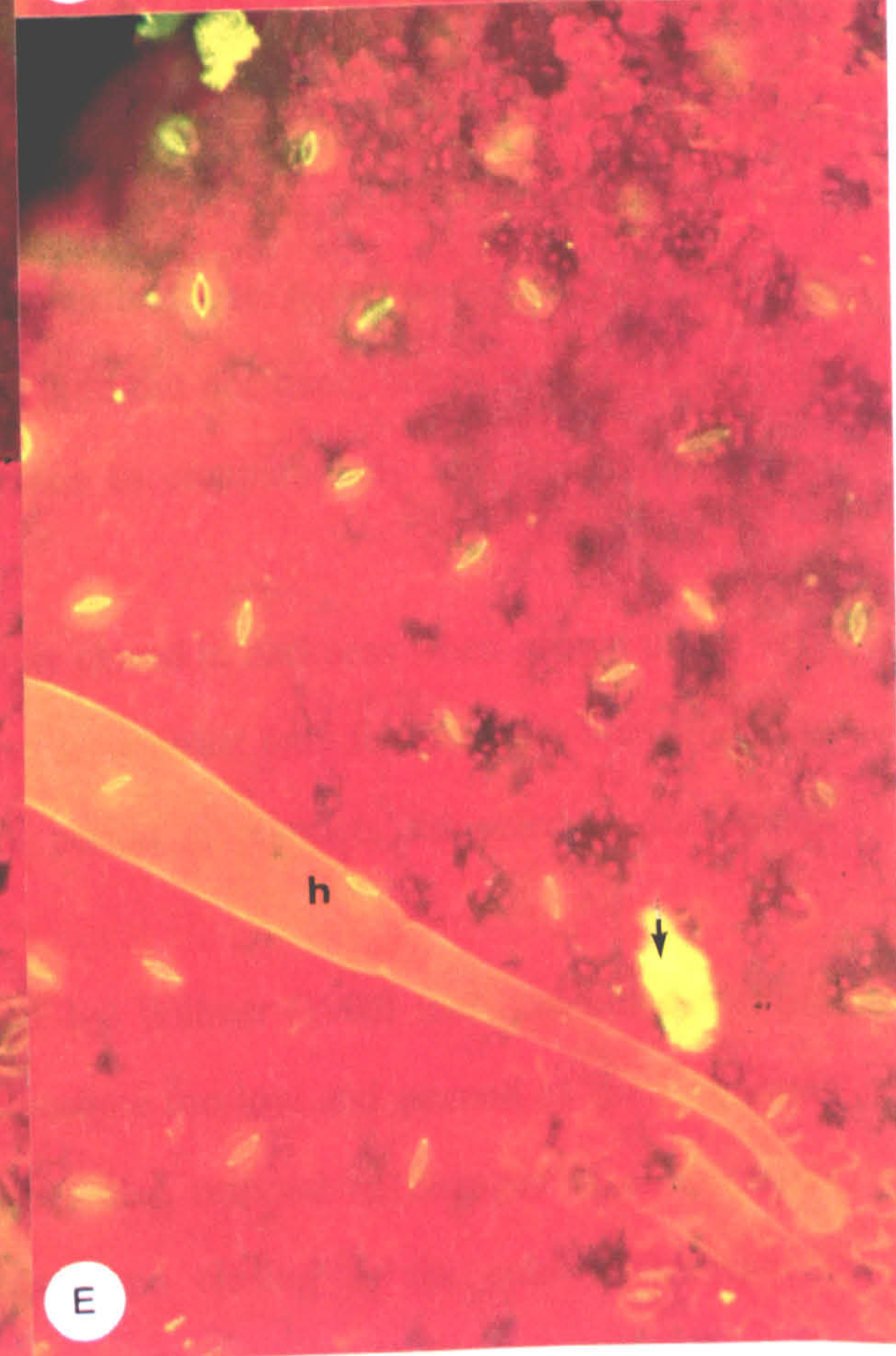
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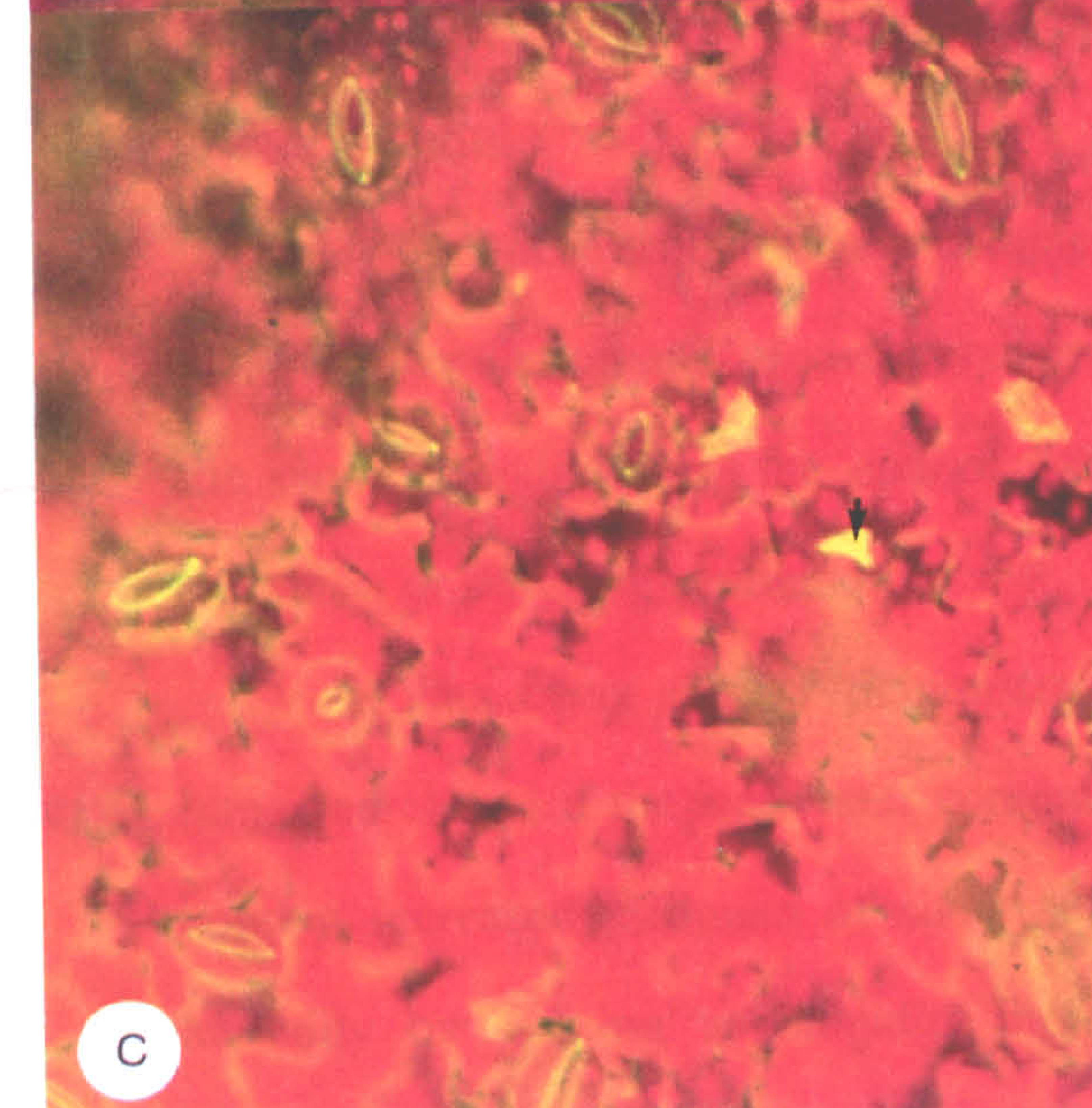

C

lengths; this was particularly obvious in cauliflower. The reverse was true of plants grown in forced ventilation or *in vivo*; (Figs. 6.06, 6.07; Plates 6.01, 6.02, 6.04).

In the sealed and diffusive treatments stomata of both tobacco and cauliflower remained widely open in the dark as well as in the light period (Figs. 6.04 and 6.05) as a consequence larger pore area were noticed in the sealed condition in cauliflower and tobacco respectively in darkness. The result indicates that the normal functioning of stomata was inhibited or that the stomata failed to mature to a normally functioning state. The non-functional stomata of *in vitro*-grown leaves of apple and plum from conventional tissue culture systems were also observed by Brainerd and Fuchigami (1981, 1982) and Fuchigami, Cheng and Soldner (1981) respectively.

On the other hand, stomata from the fast flow and *in vivo* treatments appeared to function normally and to close in darkness. Stomata of the plantlets grown under forced ventilation (fast and very fast flow), and also particularly those grown in the *in vivo* condition, exhibited remarkably lower pore areas. In these treatments the measured pore areas were relatively larger in light and smaller in darkness which points out the normal functioning of stomata in these treatments. Furthermore, it was very noticeable that the stomata from the *in vivo* treatments in the light did not gape, but appeared to be only partially open. This may well have been due to comparatively low RH (30%) in the growth room

Most of the stomata were completely closed in darkness when grown under very fast flow ventilation and *in vivo* conditions. In contrast only few stomata were found in the closed condition by the end of dark period in plantlets subjected to the sealed condition or diffusive ventilation.

It has been already demonstrated by Willmer (1983) that generally stomata remained closed if the leaf water content became too low and opened as the leaf water content increased. Stomata of some species also respond to changes in atmospheric humidity by opening as humidity increases and closing as the culture atmosphere becomes drier (Watts and Neilson 1978). These results accord with the present findings of cauliflower and tobacco plantlets which showed widely open stomata under sealed

conditions or with diffusive ventilation, possibly as a response to very high RH (*ca.* 100%) in the culture vessels, but some normally functioning stomata were also observed in the plantlets grown under very fast forced ventilation.

### 6.3.3. Leaf Internal Anatomy

#### 6.3.3.1. Cauliflower

Transverse sections of leaves from the sealed condition often showed a lack of well defined palisade and spongy mesophyll layers (Plate 6.06). In all the tissues of the leaf the cells were very small; in the mesophyll layers the cells were more closely packed and with smaller intercellular spaces than those of the other treatments

In contrast, leaves from plantlets subjected to diffusive or forced ventilation and from those *in vivo* had definite palisade and spongy mesophyll layers, the latter with large intercellular spaces; all the cells were generally much larger than those from the sealed treatment.

#### 6.3.3.2. Tobacco

There were obvious differences in internal anatomy between leaves from the sealed and diffusive treatments and those from the forced ventilation and *in vivo* treatments after 28 days (Plate 6.07). The leaves from the former two treatments were senescing, and as a consequence, (a) the cells may have already been disrupted, or (b) the cells may have been delicate and comparatively easily distorted by the preserving and embedding processes. The sections indicate that leaf thickness was greatest in the *in vivo* treatment and that there was more structural integrity in the leaves from this and from the forced ventilated treatments. The sections also suggest that the chloroplast contents of the mesophyll layers in these treatments were much greater than those from the sealed and diffusive treatments. This would agree with the results in which the chlorophyll contents of the leaves were compared (Chapter V).

### 6.3.4. Epicuticular and cuticular waxes in cauliflower

Plantlets developed under different treatments exhibited different degrees of epicuticular wax which were also observed under the microscope. The leaves from *in vivo* grown plantlets and FF-ventilation treatment (*e.g.* Plate 6.04D), and to some degree from the SF-ventilation treatment showed intense epicuticular wax development which was evident as a white powdery coating and gave the leaves a blue-green colour (Plate 6.01H and I); these effects were absent from plants in the sealed and diffusive treatments.

These observations agree with earlier investigations which showed that regenerated cauliflower plantlets did not have any epicuticular wax; this normally develops during the hardening period (Grout 1975). It was also reported by Grout (1975) that the leaves from cauliflower culture and also from the plantlets grown in culture vessels showed less epicuticular wax when compared with seedlings grown in the green house or culture room. The development of epicuticular wax is already known to be advantageous for the plantlets during the acclimatisation period (Grout 1975; Sutter and Langhans 1982); it probably helps to protect the plants from desiccation.

The leaves from the sealed and diffusive treatments also exhibited thinner layers of cuticular waxes (Plate 6.08). Also in the present study, in plantlets grown *in vivo* or with forced ventilation, the cuticular waxes were more obvious and fluoresced more in blue light, especially when compared to those of plants grown in diffusive ventilation.

It seems possible that these effects were responses to the lower humidities in the forced ventilation and especially in the *in vivo* treatments; the higher rates of photosynthesis may also have played a part by facilitating the production of lipids .

### 6.3.5. Leaf hairs in tobacco

In all treatments the lengths and densities of leaf epidermal hairs varied according to their position on the leaf (Fig. 6.08).

Epidermal hairs were shortest on shoots in the sealed treatment and increased in length with increasing efficiency of ventilation. For example, comparing those from the basal mid-rib region, they were at least 2.6X as long in very fast flow ventilation and 4X

## PLATE : 6.06

**Cauliflower:** transverse sections of 3rd or 4th leaves from apex of plantlets grown under different types of ventilation and also *in vivo* condition for 28 days as indicated bellow (X160).

(A) sealed condition (silicone rubber bung); note lack of well defined palisade and spongy mesophyll layers; cells are smaller and with less intercellular space.

(B) diffusive ventilation (polypropylene disc).

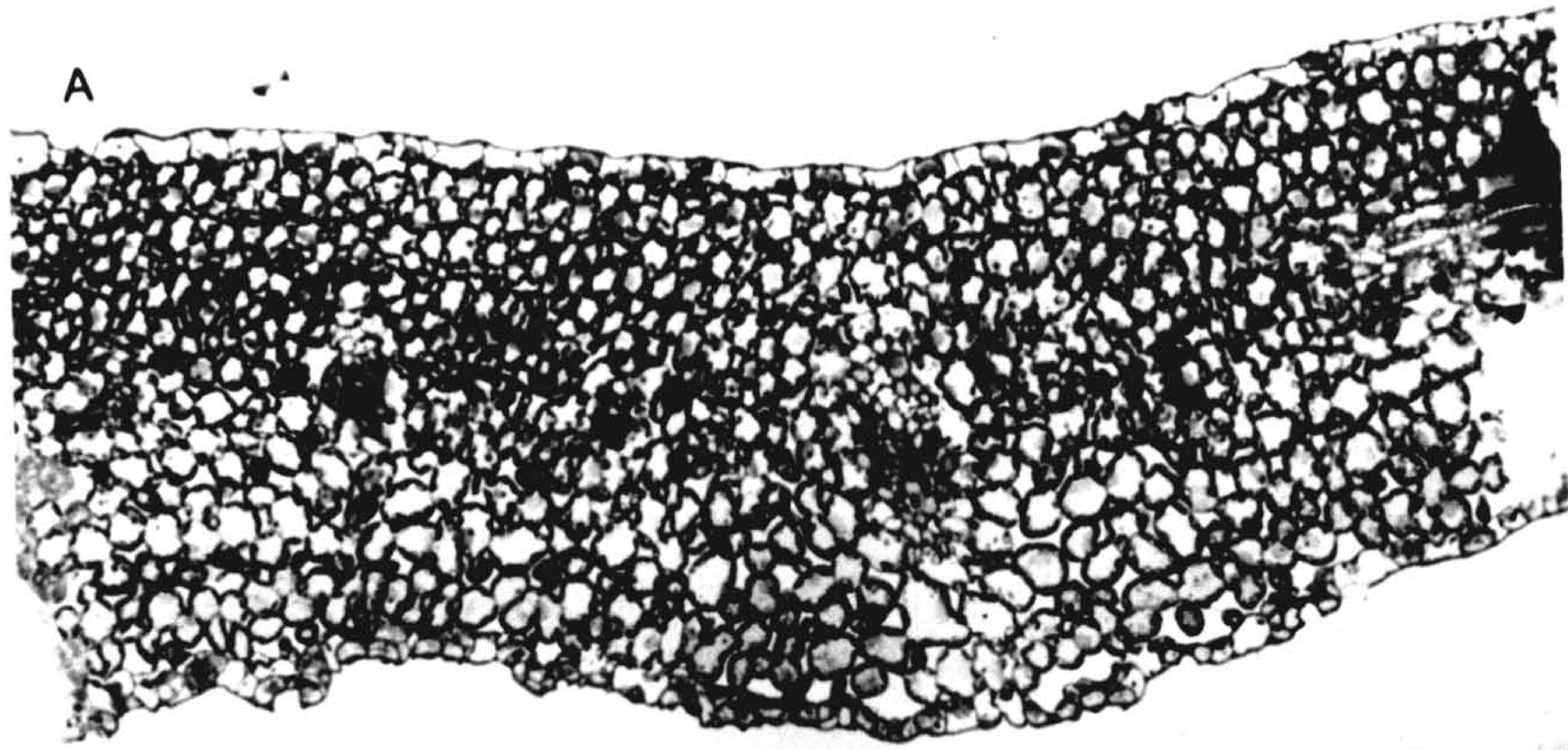
(C) fast forced ventilation (5 $cm^3$ $min^{-1}$).

(D) very fast forced ventilation (flow rate was 10 $cm^3$ $min^{-1}$ ).

(E) *in vivo* (growth room conditions).

Note in B, C, D and E the palisade and spongy mesophyll layers are well defined and with large intercellular spaces in the spongy mesophyll.

Culture vessel volume was 60 $cm^3$. Cultures were grown at *ca.* 25°C with 8 hours dark and 16 hours light periods ; PAR = 150 µmol $m^{-2}s^{-1}$.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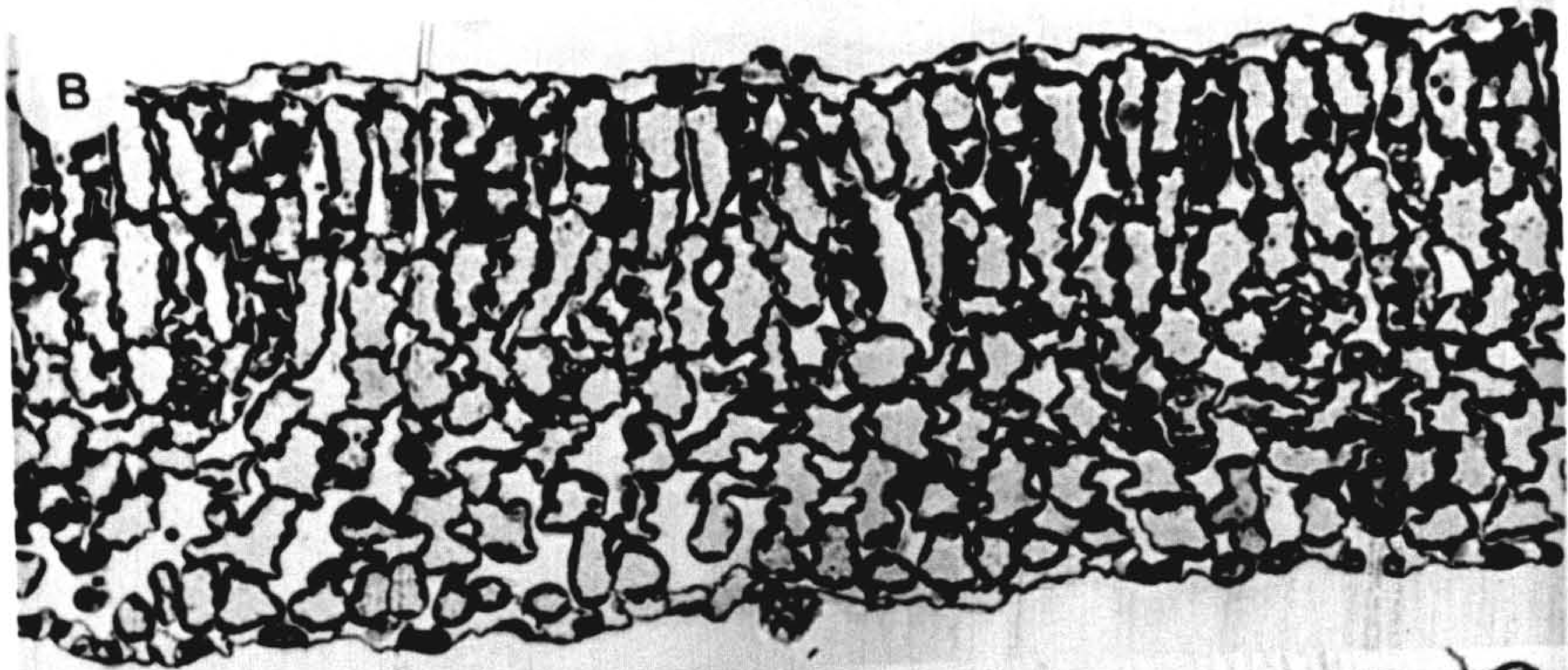
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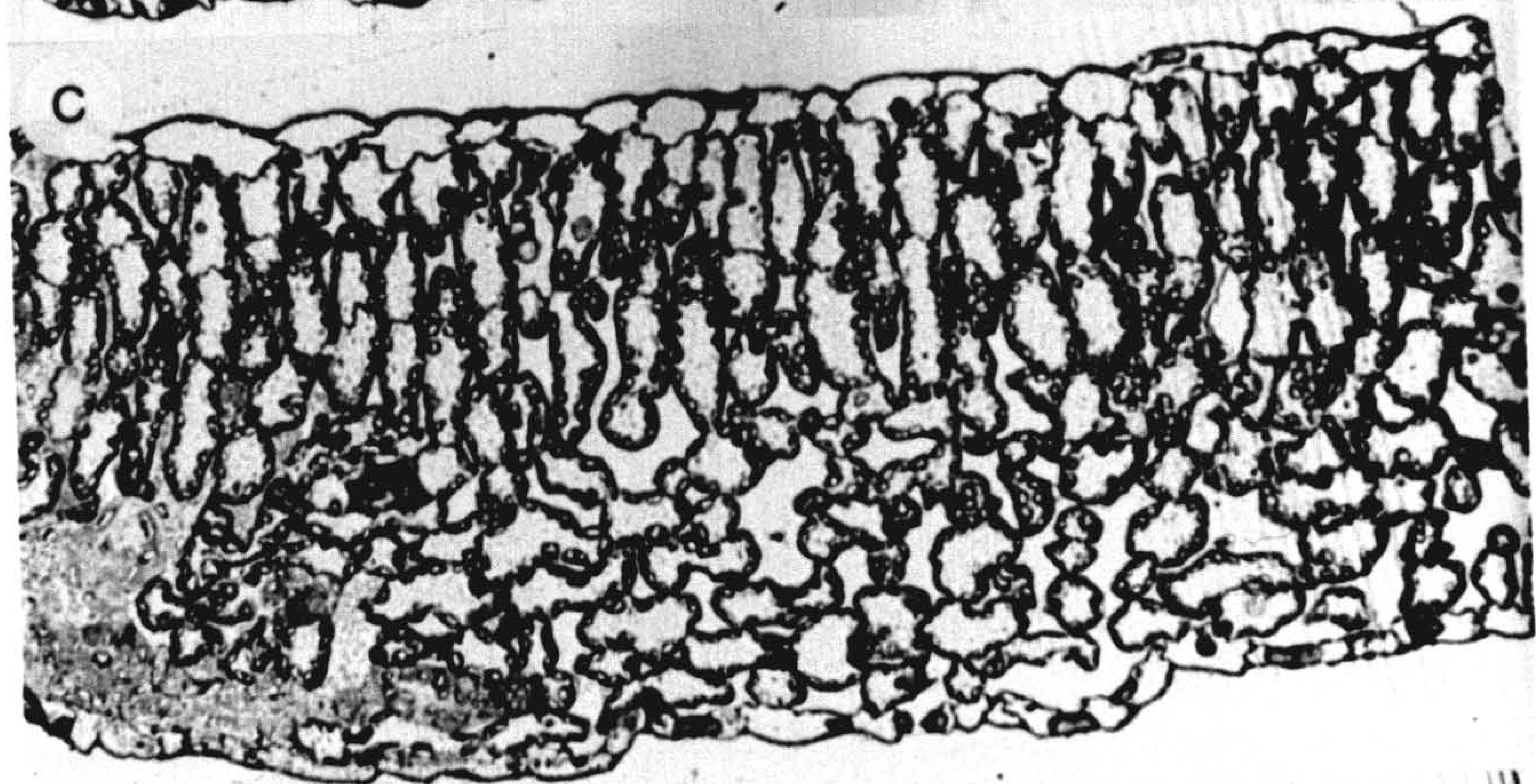
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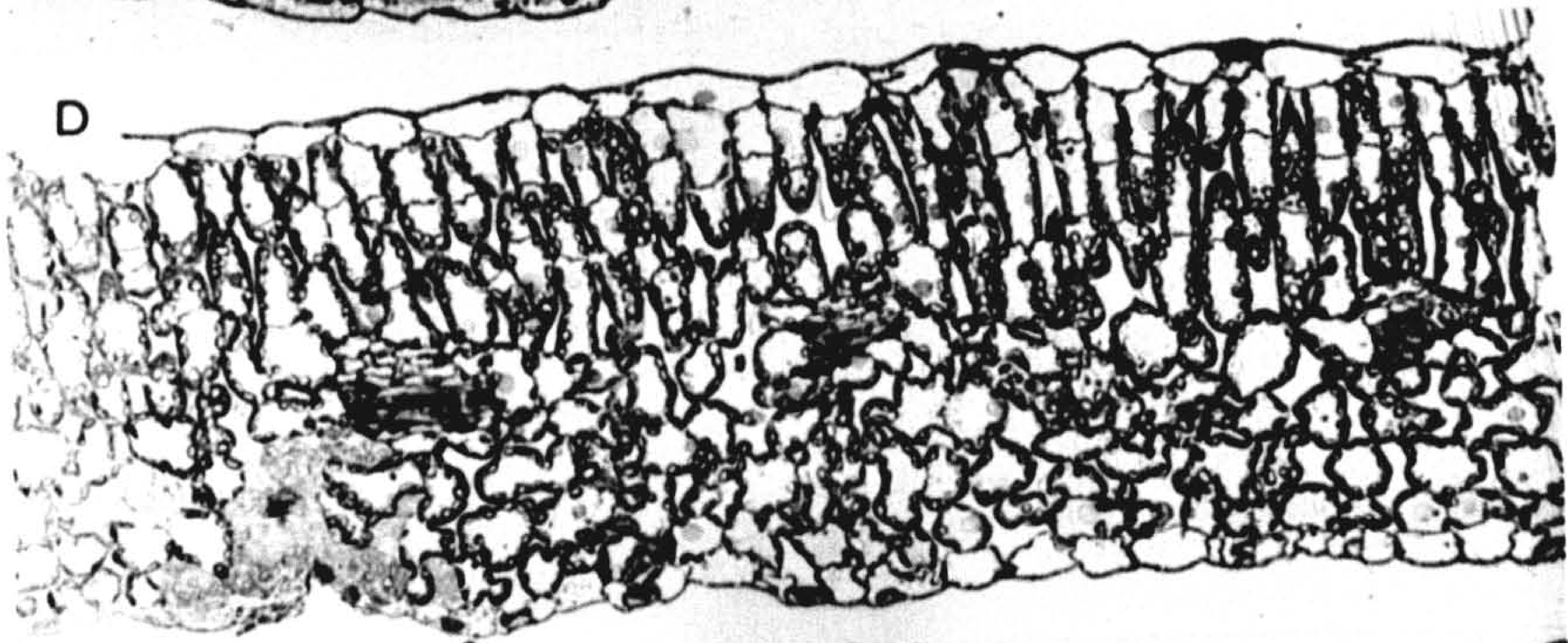
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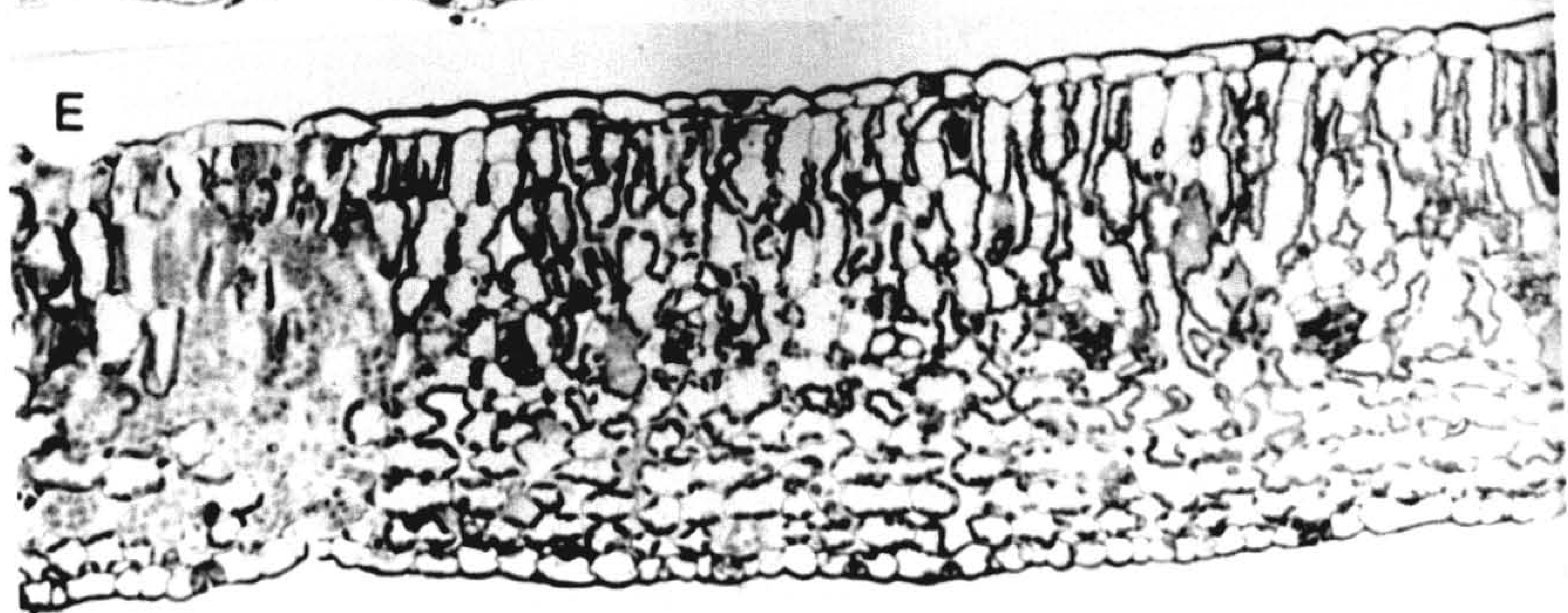
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## PLATE : 6.07

**Tobacco:** transverse sections of 3rd or 4th leaves from apex of plantlets grown under different types of ventilation and also *in vivo* condition for 28 days as indicated bellow (X160)

(A) sealed condition (silicone rubber bung).

(B) diffusive ventilation (polypropylene disc).

(C) fast forced ventilation (5 $cm^3$ $min^{-1}$).

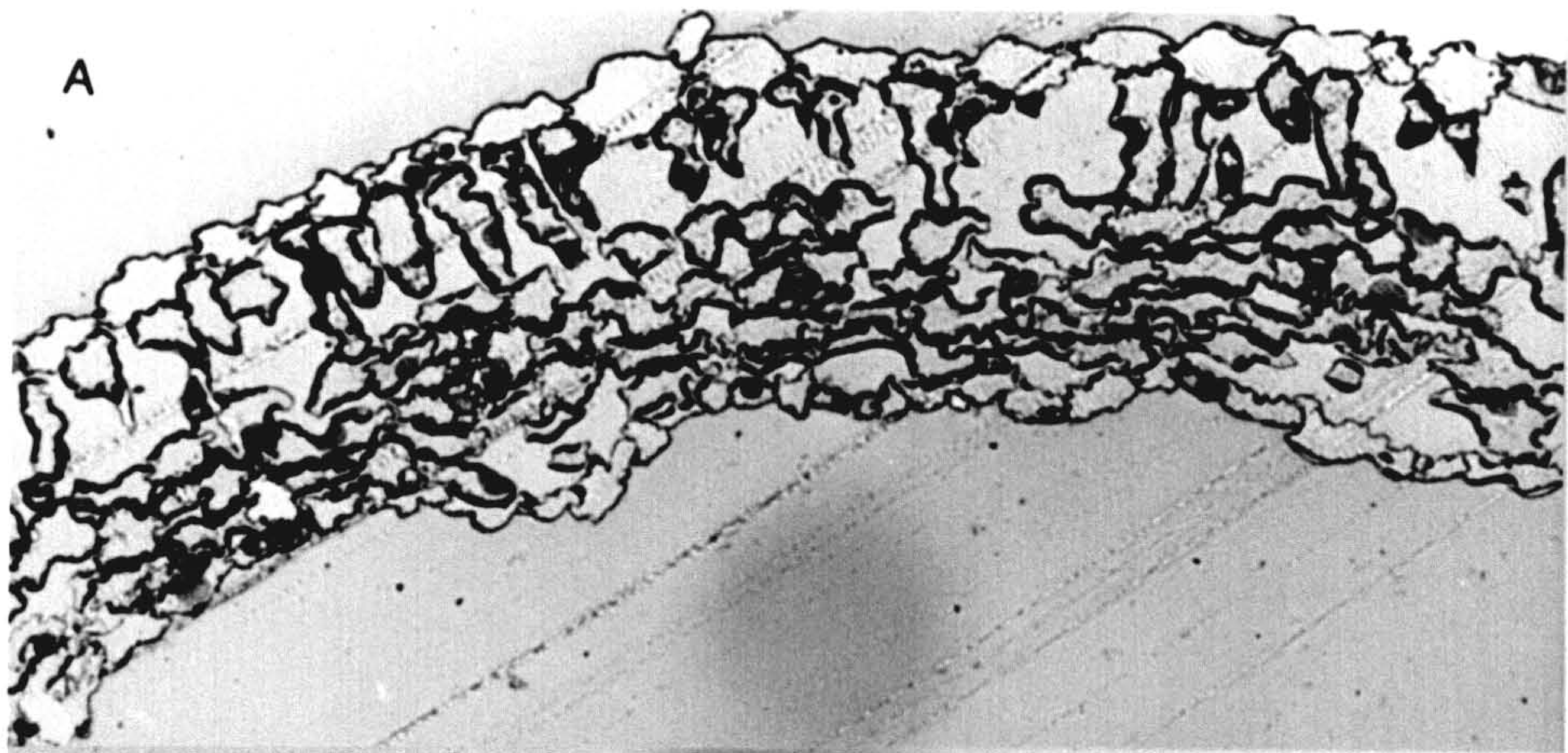
(D) very fast forced ventilation (flow rate was 10 $cm^3$ $min^{-1}$ )

(E) *in vivo* (growth room conditions).

Note in A and B the cells appear disrupted and with very large spaces in the palisade layers. This may have been a consequence of the senescence of the leaves and / or the delicate nature of the cells, which made them more easily distorted by the preserving and the embedding processes. Also the chloroplasts are not very obvious.

Note in C, D and E cell integrity was preserved, the cells are not disrupted and the chloroplasts are obvious. The leaf thickness in the *in vivo* treatment was markedly greater than in the other treatments.

Culture vessel volume was 60 $cm^3$. Cultures were grown at *ca.* $25^0C$ with 8 hours dark and 16 hours light periods ; PAR = 150 μmol $m^{-2}s^{-1}$.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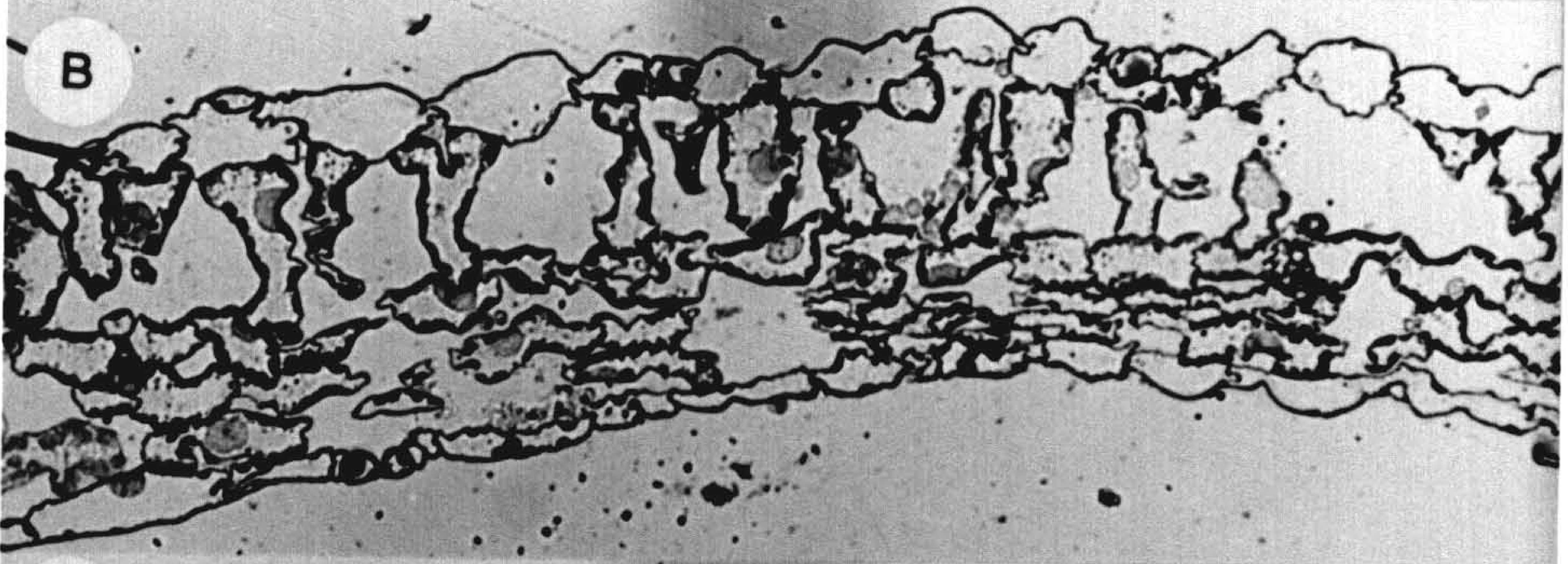
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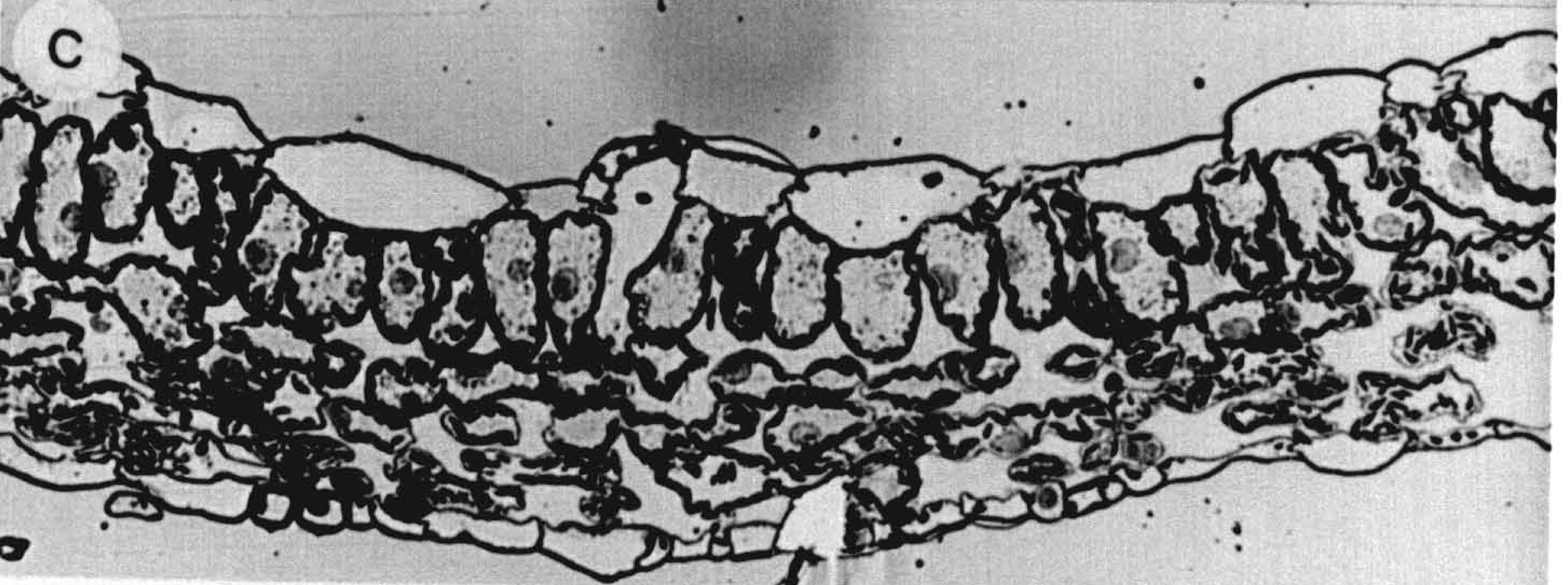
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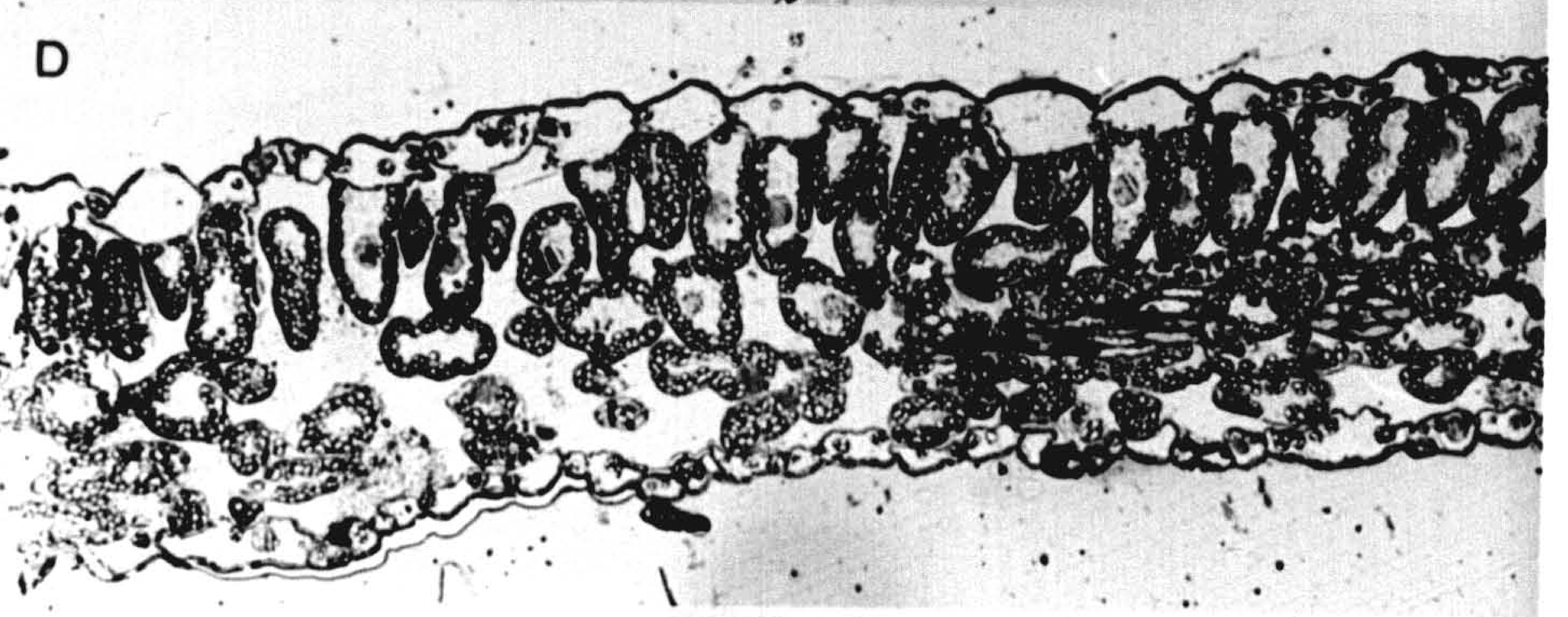
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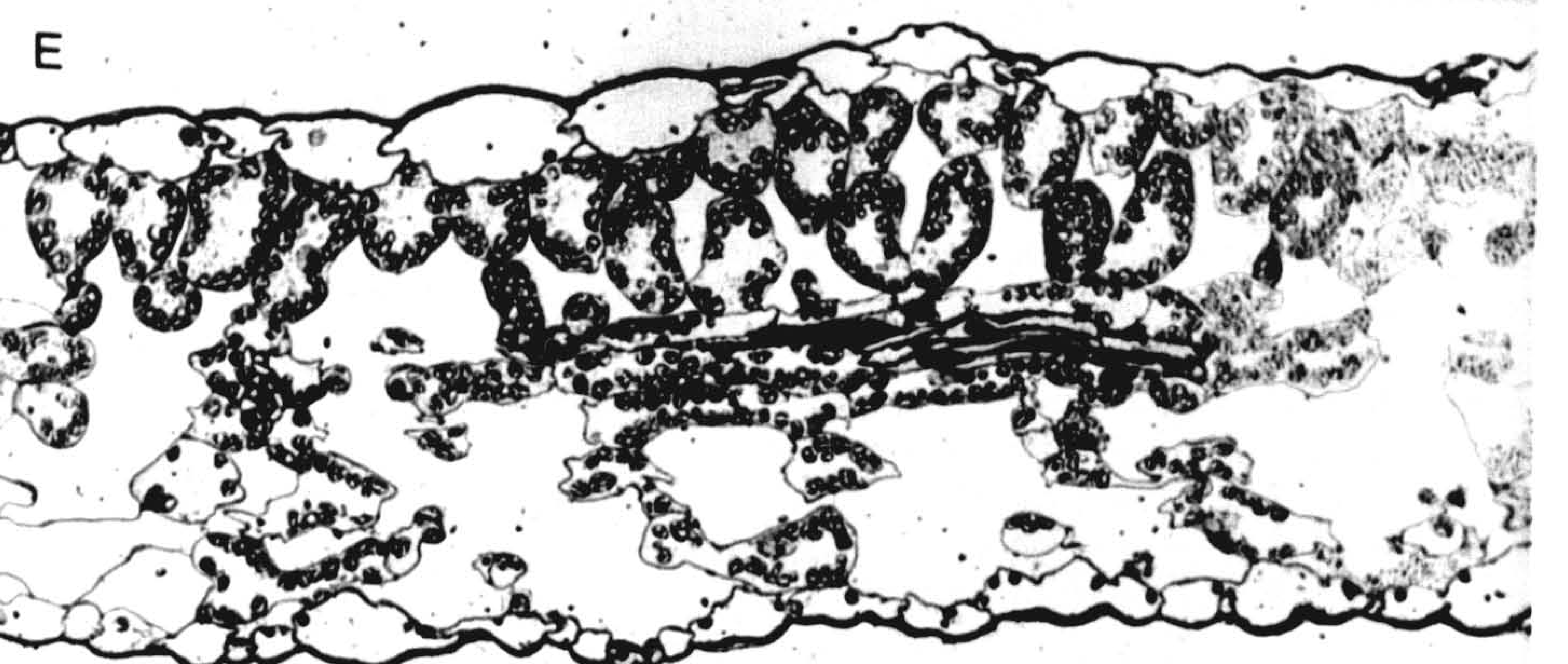
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## PLATE : 6.08

**Cauliflower :** Transverse sections of upper epidermis of fresh 3rd or 4th leaf from apex of 28 days old plantlets; sections were stained in 0.02% aqueous auramine and photographed under blue light to show waxes fluorescing yellow. Culture vessel volume was 60 $cm^3$. Growth room conditions : *ca.* 25°C with 8 hours dark and 16 hours light periods; PAR = 150 μmol $m^{-2}s^{-1}$; RH = 26 - 32%. Plantlets were grown under different types of ventilation and also *in vivo* as indicated bellow (X688).

(A) sealed condition (silicone rubber bung).

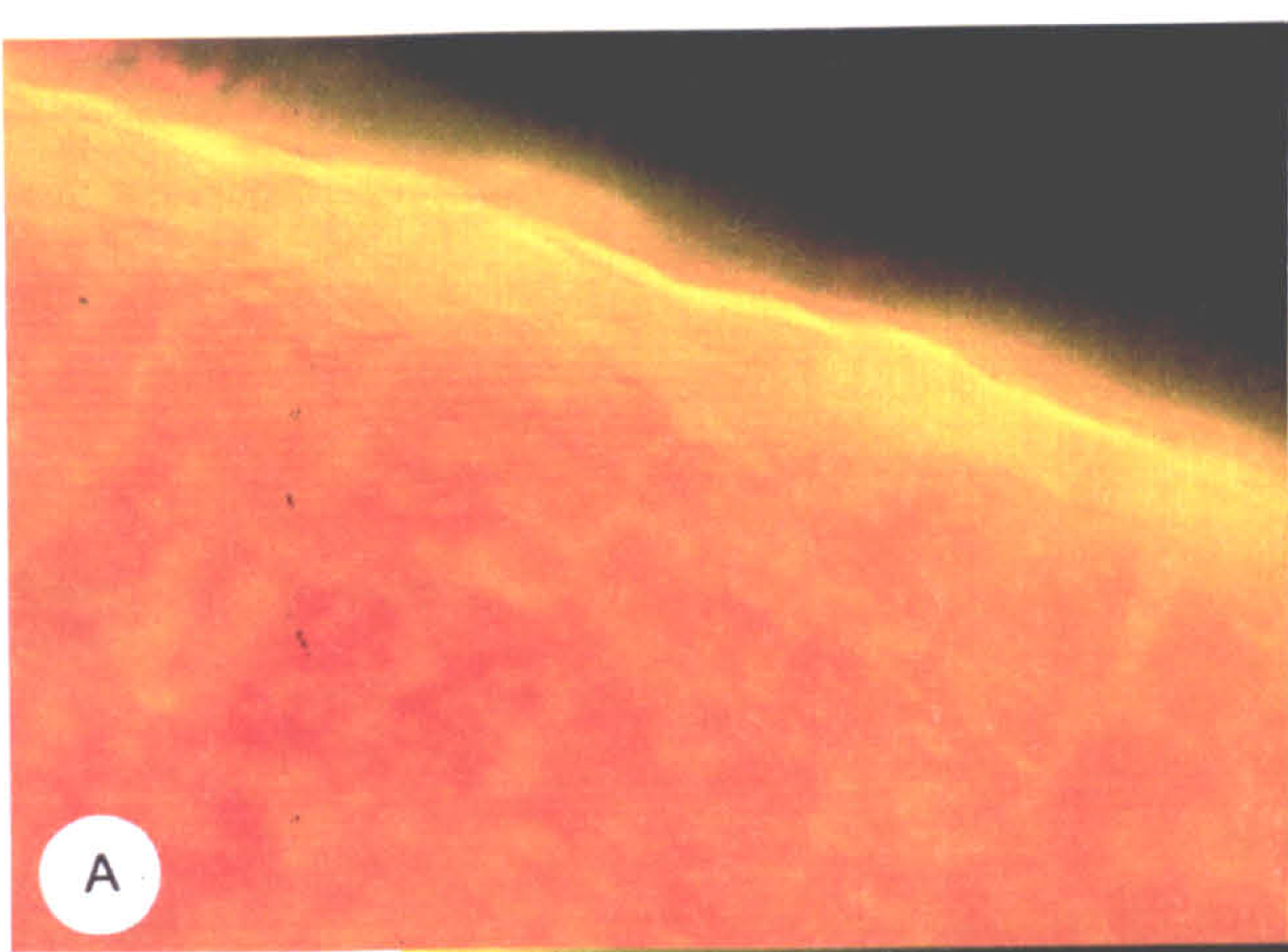
(B) diffusive ventilation (polypropylene disc).

(C) fast forced ventilation (flow rate was 5 $cm^3$ $min^{-1}$).

(D) very fast forced ventilation (flow rate was 10 $cm^3$ $min^{-1}$).

(E) *in vivo* (growth room conditions).

Note in (A) and (B) the cuticles appeared thinner and fluoresced to a smaller degree than in (C), (D) and (E). The lack of fluorescence was particularly obvious in (B).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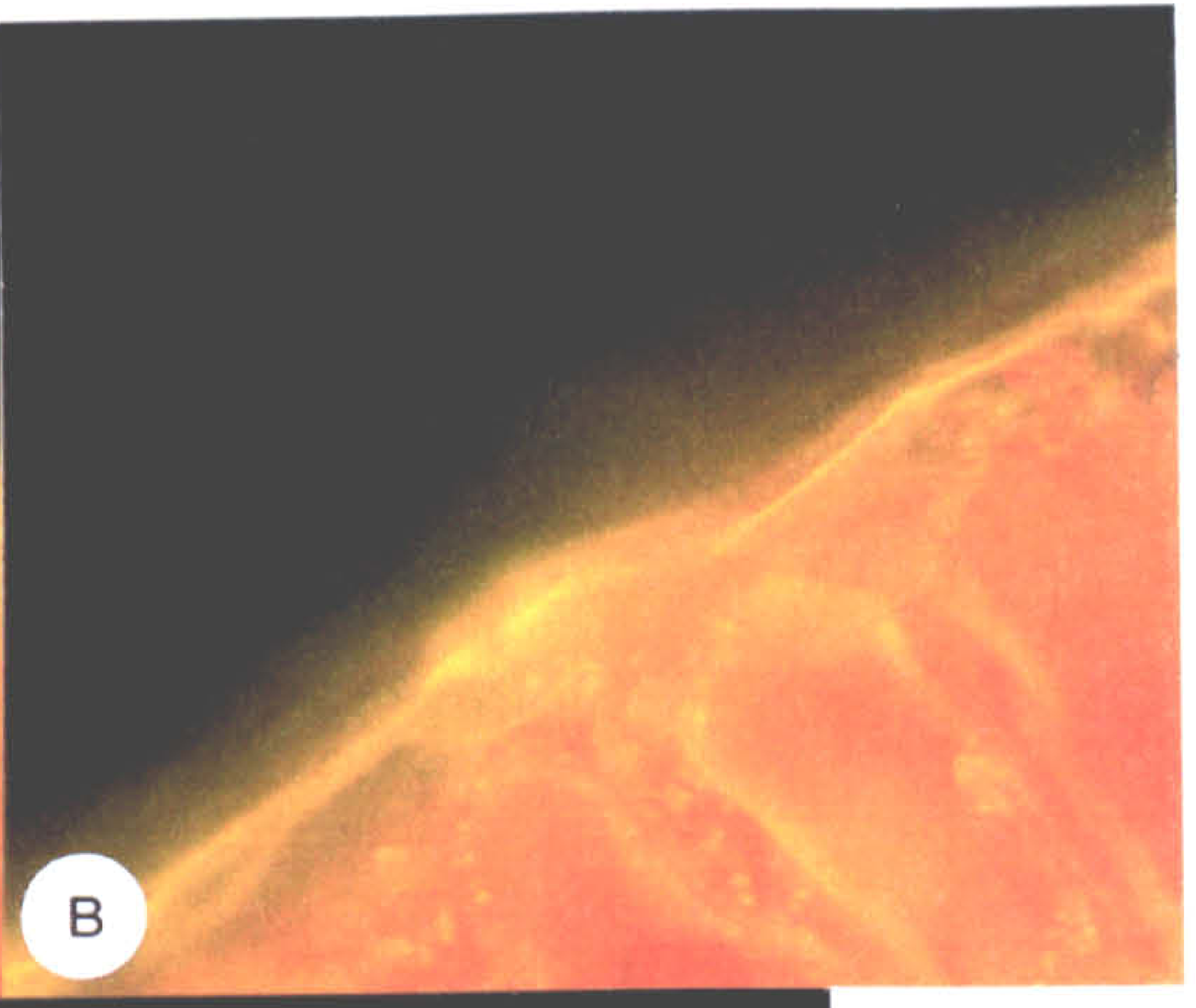
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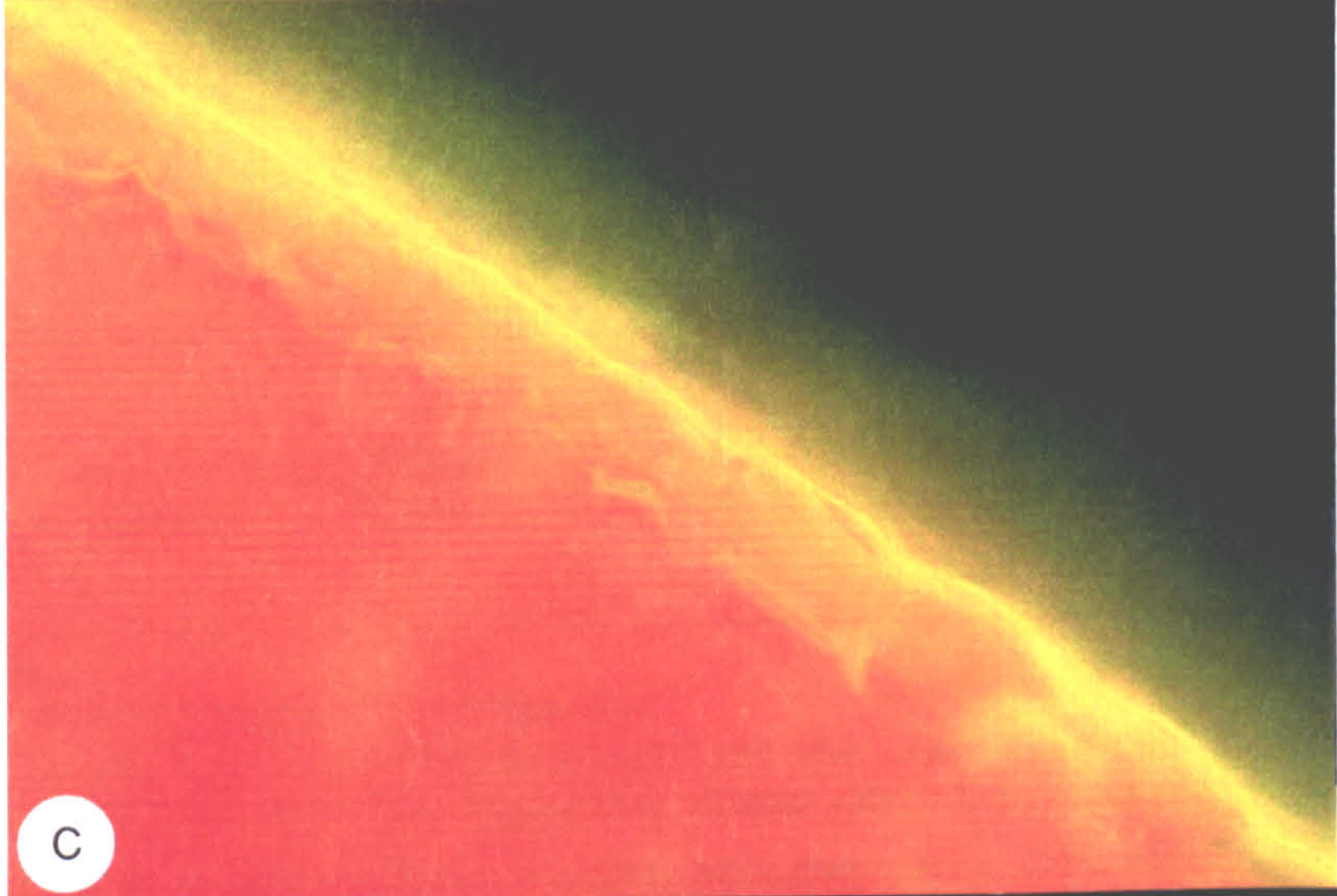
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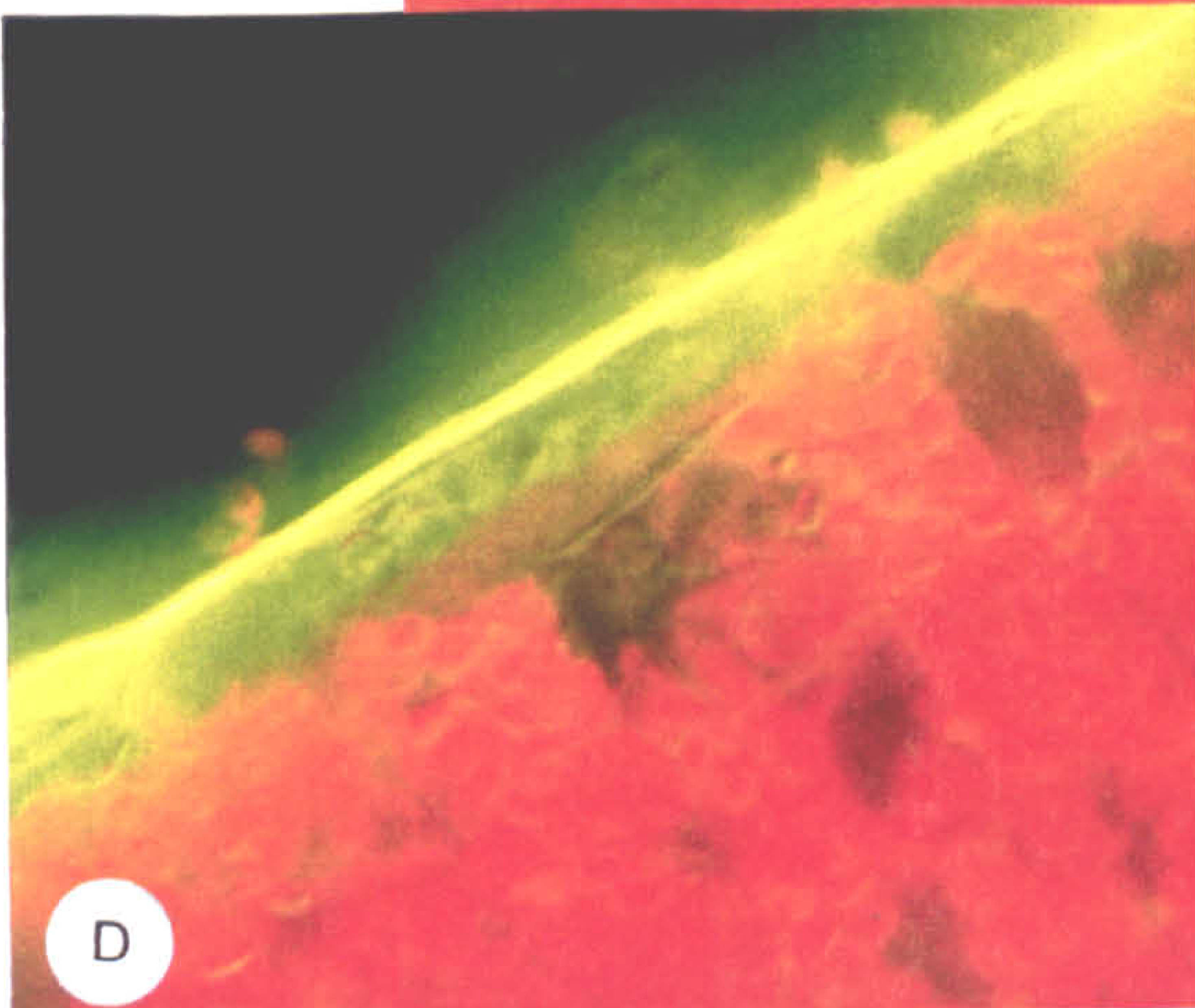
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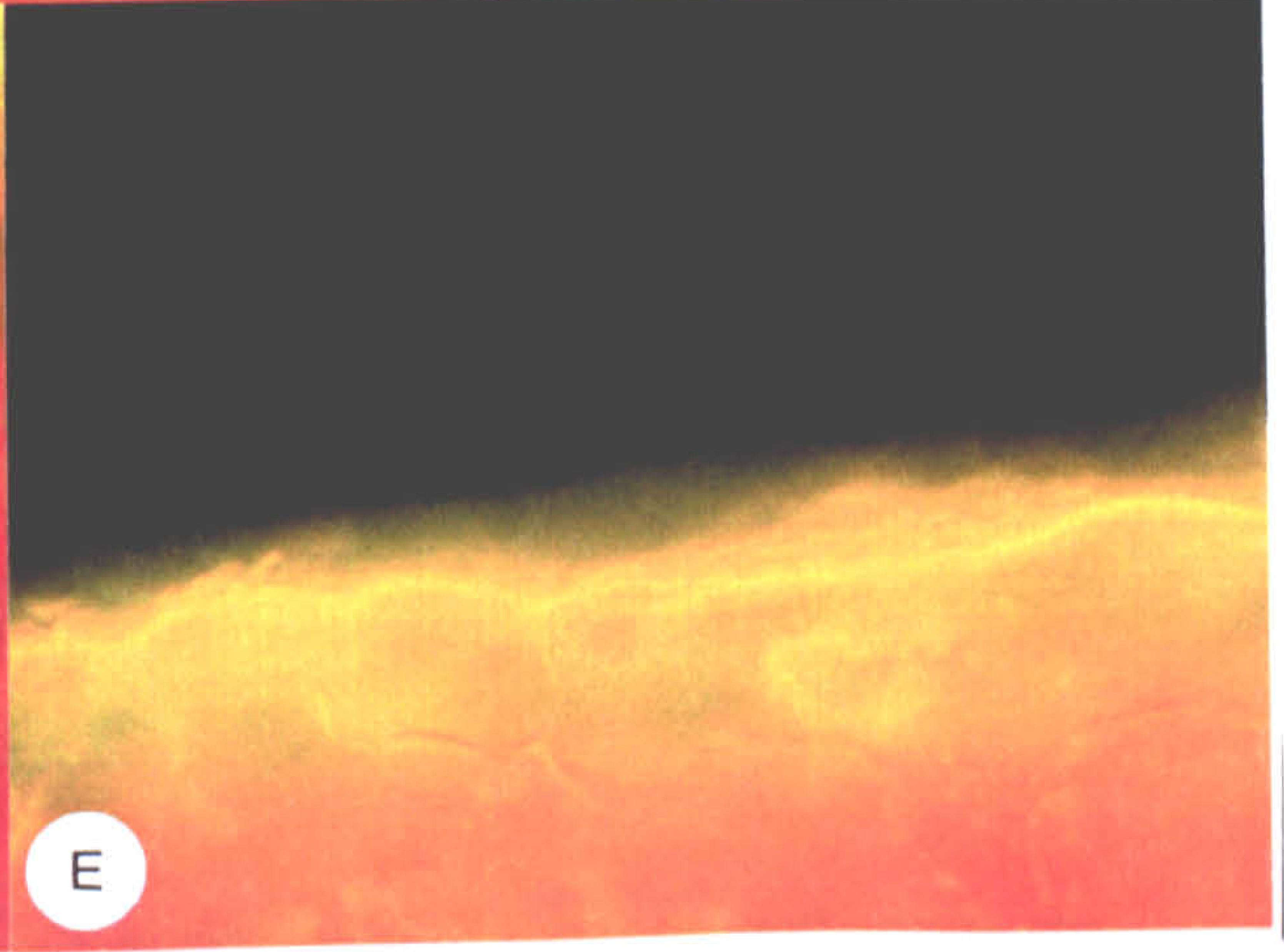
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**Table 6.01.** Tobacco : measurement of hairs from the lower (abaxial) side of leaves (3rd or 4th); plantlets developed *in vivo* and *in vitro* (sealed, diffusive and forced ventilation).

| Ventilation | Characteristics | Region A | Region B | Region C | Region D |
|---|---|---|---|---|---|
| Sealed | Length (µm) | 261 ± 51 | 503 ± 98 | 350± 24 | 135 ± 11.0 |
| | Number (per $mm^2$ leaf area) | 19.9 ± 2.1 | 34 ± 5.0 | 75 ± 9.0 | 12.8 ± 0 8 |
| Diffusive ventilation | Length (µm) | 351 ± 41 | 710± 85 | 625 ± 51 | 232.5 ± 21 |
| | Number (per $mm^2$ leaf area) | 11.8 ± 2.0 | 18.1 ± 2.0 | 77.1 ± 8 3 | 4 8 ± 0.9 |
| Forced ventilation (fast) | Length (µm) | 461 ± 51 | 862.5 ± 49 | 725 ± 23 | 311 ± 32 |
| | Number (per $mm^2$ leaf area) | 7.3 ± 0.6 | 15.3 ± 1.1 | 25 1 ± 1 3 | 3 1 ± 0 7 |
| Forced ventilation (very fast) | Length (µm) | 519 ± 23 | 1329 ± 230 | 931 ± 93 | 335 5 ± 41 |
| | Number (per $mm^2$ leaf area) | 6.9 ± 0.5 | 13.9 ± 0.7 | 14.3 ± 2 1 | 2.9 ± 0.6 |
| *In vivo* | Length (µm)<br>Number (per $mm^2$ leaf area) | 601± 53<br>6.0 ± 0.3 | 2087 ± 191<br>11.2 ± 1.3 | 1075 ± 119<br>22.3 ± 1 3 | 409 ± 21<br>1 4 ± 0.4 |

*28 days old plantlets.

◆Each value represents a mean ± SE of 10 - 15 replicates.

◆Flow rates of fast and very fast flow ventilations were 5.0 $cm^3$ $min^{-1}$ and 10 $cm^3$ $min^{-1}$ respectively, for sealed and diffusive ventilation vessels were capped with silicone rubber bungs and polypropylene discs respectively. Volume of culture vessel = 60 $cm^3$. Cultures were grown at *ca.* 25°C with 8 hour dark and 16 hour light periods; PAR = 150 µmol $m^{-2}s^{-1}$. Each bar represents a mean ± SE of 20 replicates.

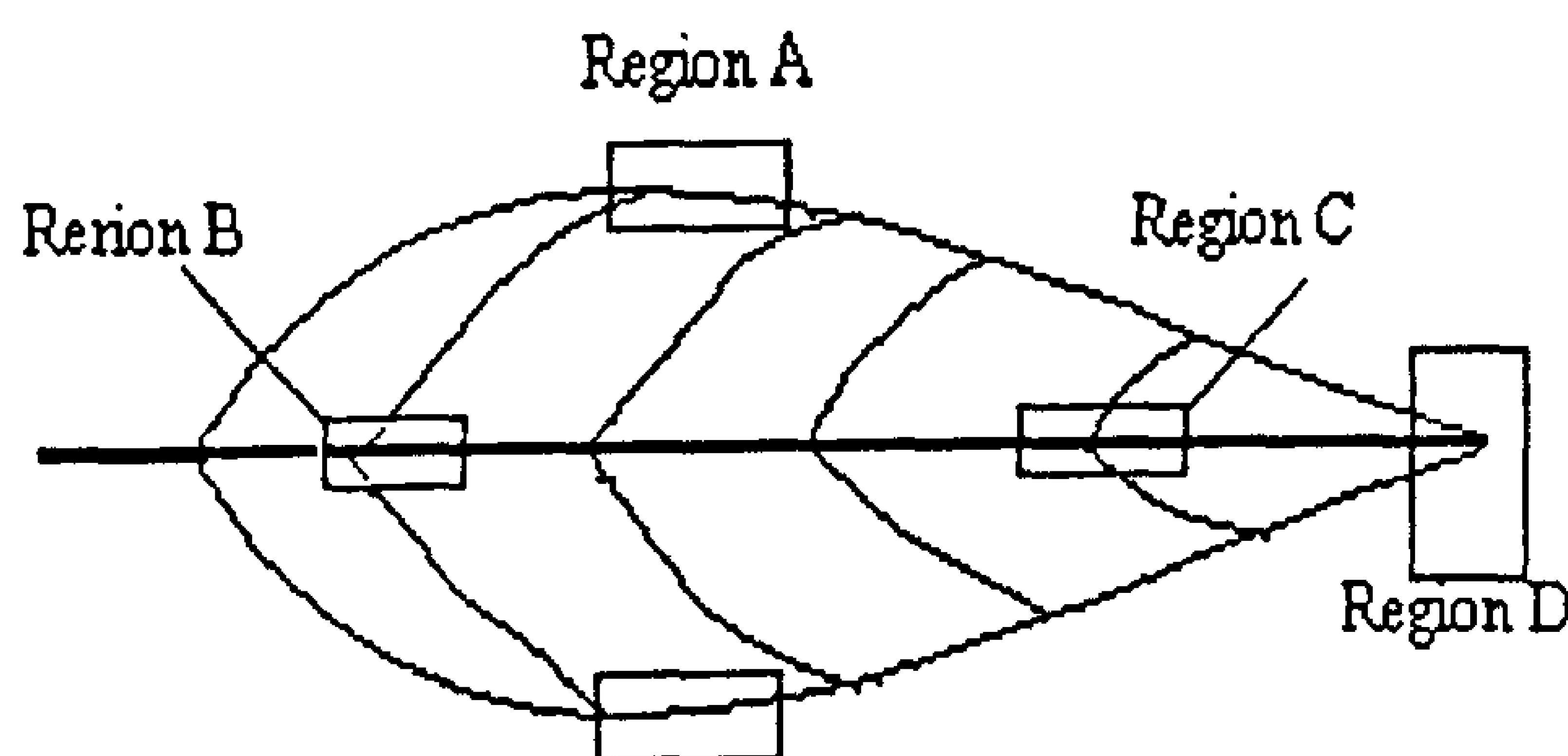



**Fig. 6.08.** Showing the regions in the lower (abaxial) side of tobacco leaf where the number and lengths of leaf hairs were measured. ( *re.* Table 6.01).

as long in the field grown plants as those from the sealed treatment (Table 6.01). This could well be a reflection of the humidity around the plantlets, hairs being longest where RH was low especially at the beginning of the experiments (Fig. 6.01). The hairs of tobacco leaves had waxy walls (Plates 6.05E, 6.09).

However, the *number* of hairs per $mm^2$ was highest in the sealed treatment, and decreased with increasing efficiency of the ventilation, being lowest in the *in vivo* grown plantlets. This could well have been an effect of the comparatively small cells in the leaves of the sealed treatment and lack of leaf expansion.

### 6.3.6 SEM: stomata in cauliflower

Note. The slower forced ventilation rate used for plants in this section was only 1.0 - 1.5 $cm^3\ min^{-1}$; the faster flow rate was 5.0 $cm^3\ min^{-1}$, this being equivalent to the "fast flow" rate used elsewhere in the chapter.

The SEM micrographs (Plates 6.10 - 6.12) highlighted the differences between treatments in respect of stomatal densities, which were found to decrease with increasing efficiency of ventilation (Figs. 6.04).

If the fixative used in preparing the specimens had indeed fixed the stomata in position, then the micrographs also illustrated the extremely open nature in the light of the pores in the sealed condition and with diffusive ventilation, compared to the partially open conditions in plants from the forced ventilation treatments (Plates 6.13 - 6.14). These same effects were also found in stomata examined from *fresh* material by light microscopy (Section 6.3.2).

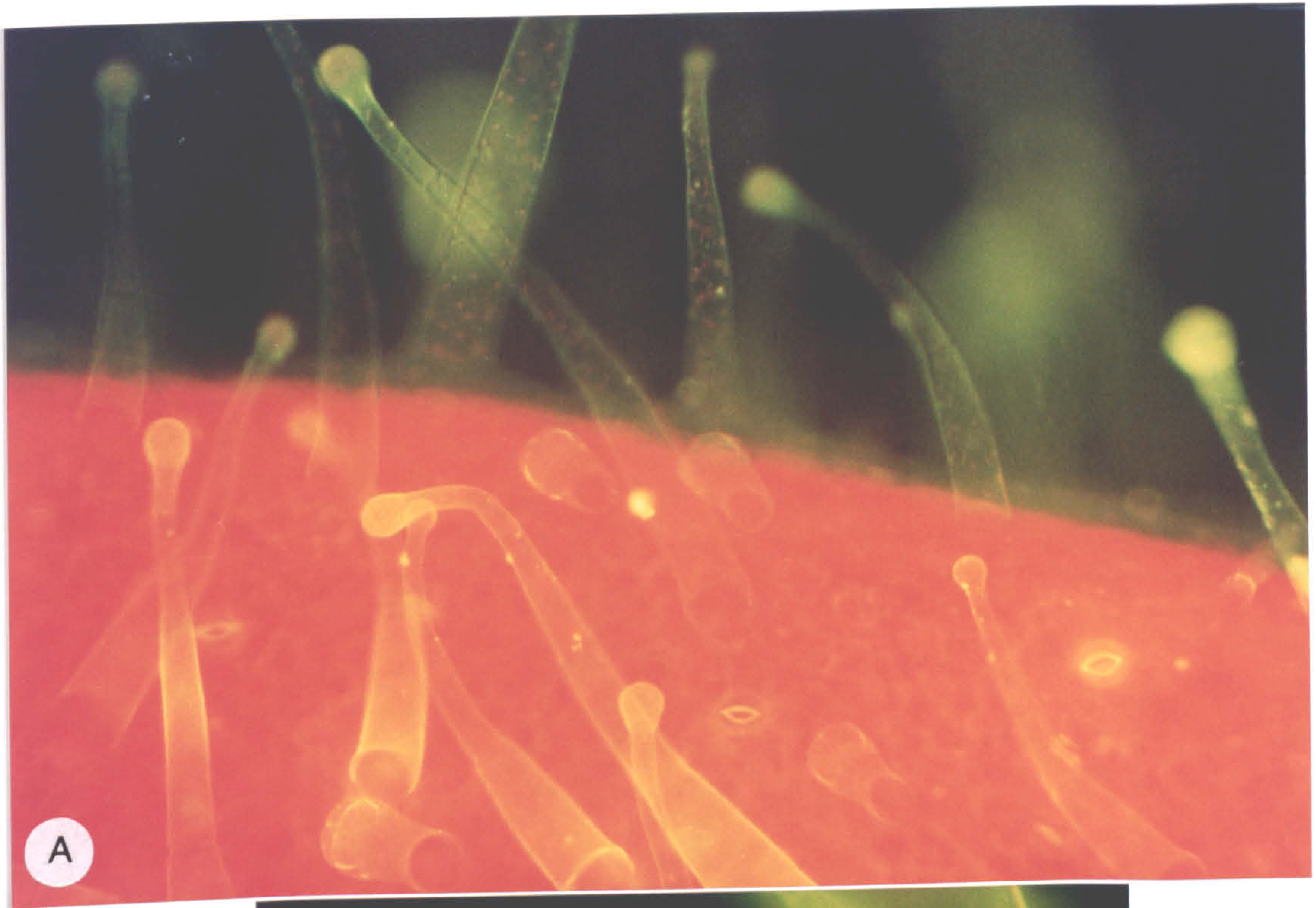
Another interesting feature of Plates 6.10, 6.11 is that the stomata from the sealed and diffusive treatments appeared to be in "elevated" positions, whereas those from the forced ventilation treatments (Plate 6.12) appeared to be slightly "sunken". These effects were also reported in Section 6.3.2. Furthermore, the degree of epidermal cell organisation and expansion appeared to be greater with increasing efficiencies of ventilation.

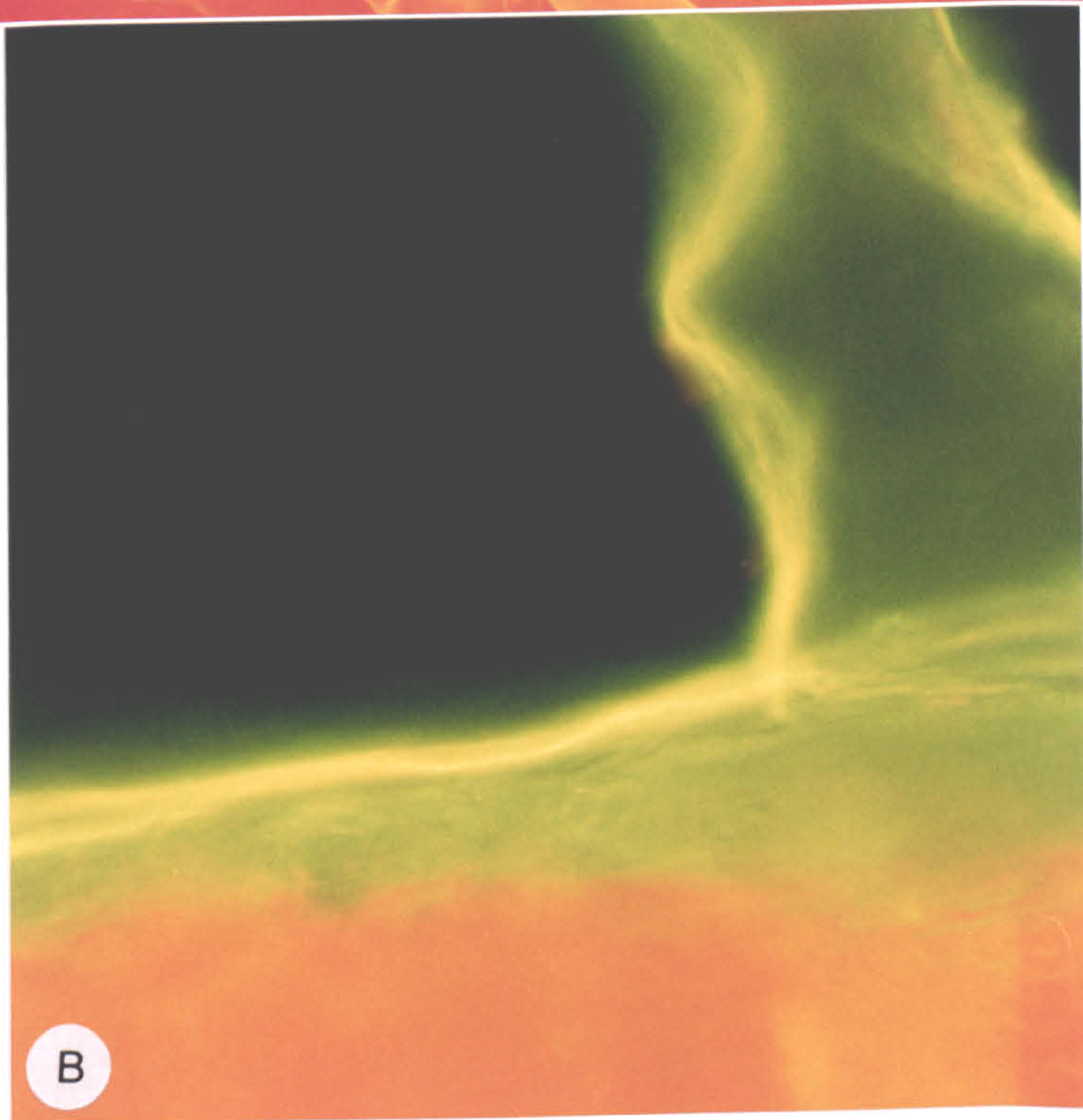
## PLATE : 6.09

**Tobacco :** Features of upper epidermis of fresh 3rd or 4th leaf from apex of 28 days old plantlets grown in forced ventilation (very fast flow; rate = 10 $cm^3$ $min^{-1}$). Specimens stained in 0.02% aqueous auramine and photographed under blue light to show waxes fluorescing yellow (also chlorophyll fluorescing red).

(A) Epidermal hairs have waxy walls and globular tips; note that the hairs are waxy at the tip (X150).

(B) Enlarged base of hair from (A) and note also fluoresce of cuticle of epidermal cells; the wall of the hair is also waxy (X688).

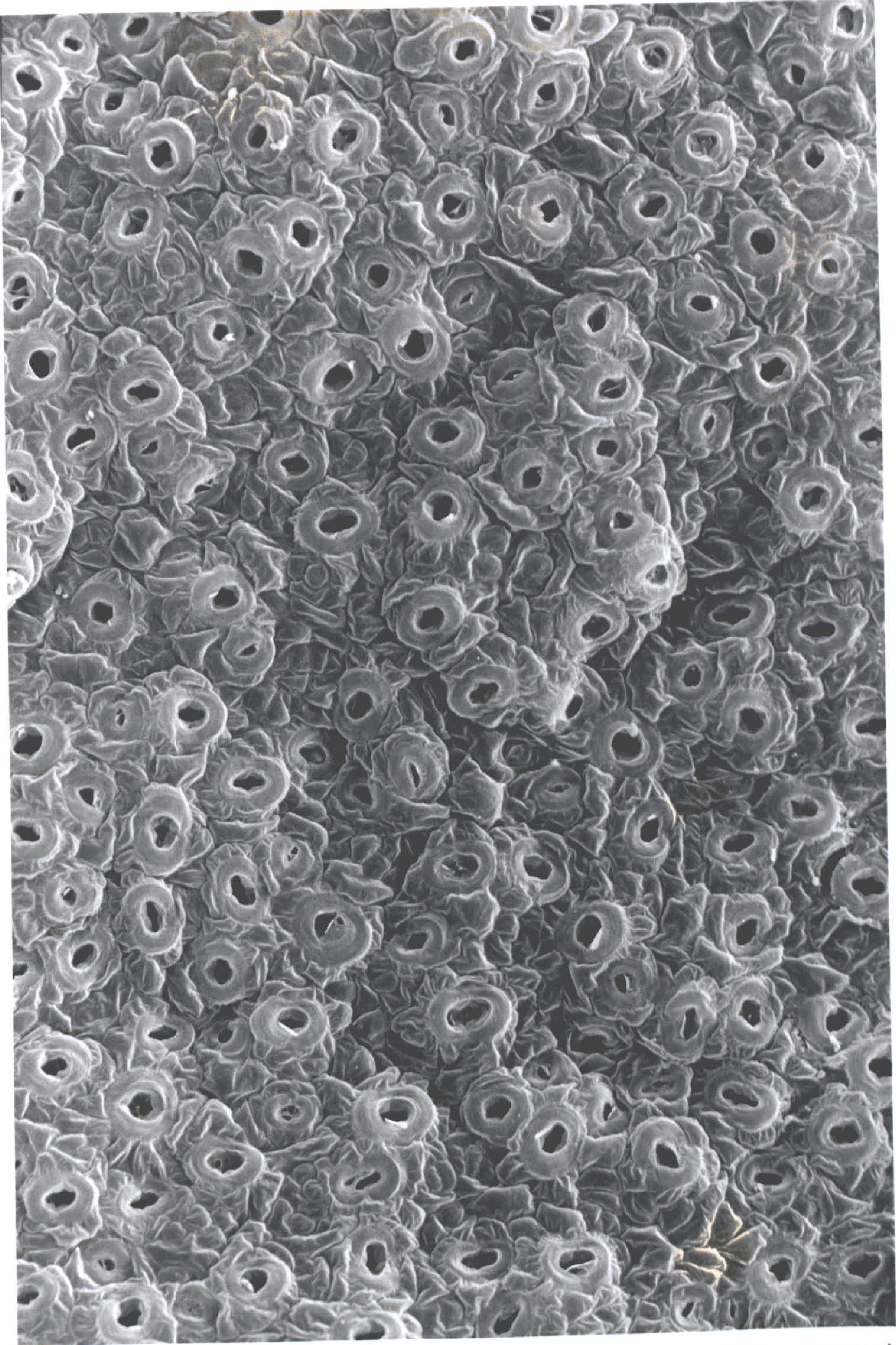

A


B

## PLATE : 6.10

**Cauliflower :** scanning electron micrograph of lower epidermis of 3rd leaf from apex of 15 days old plantlet grown in continuous light (PAR = 150 μmol $m^{-2}s^{-1}$) and in the sealed condition.

Note large stomata, densely arranged and mostly gaping widely open. Stomata appeared to be in 'elevated' positions; some, perhaps immature ones, were quite small and relatively closed.


0.1 mm

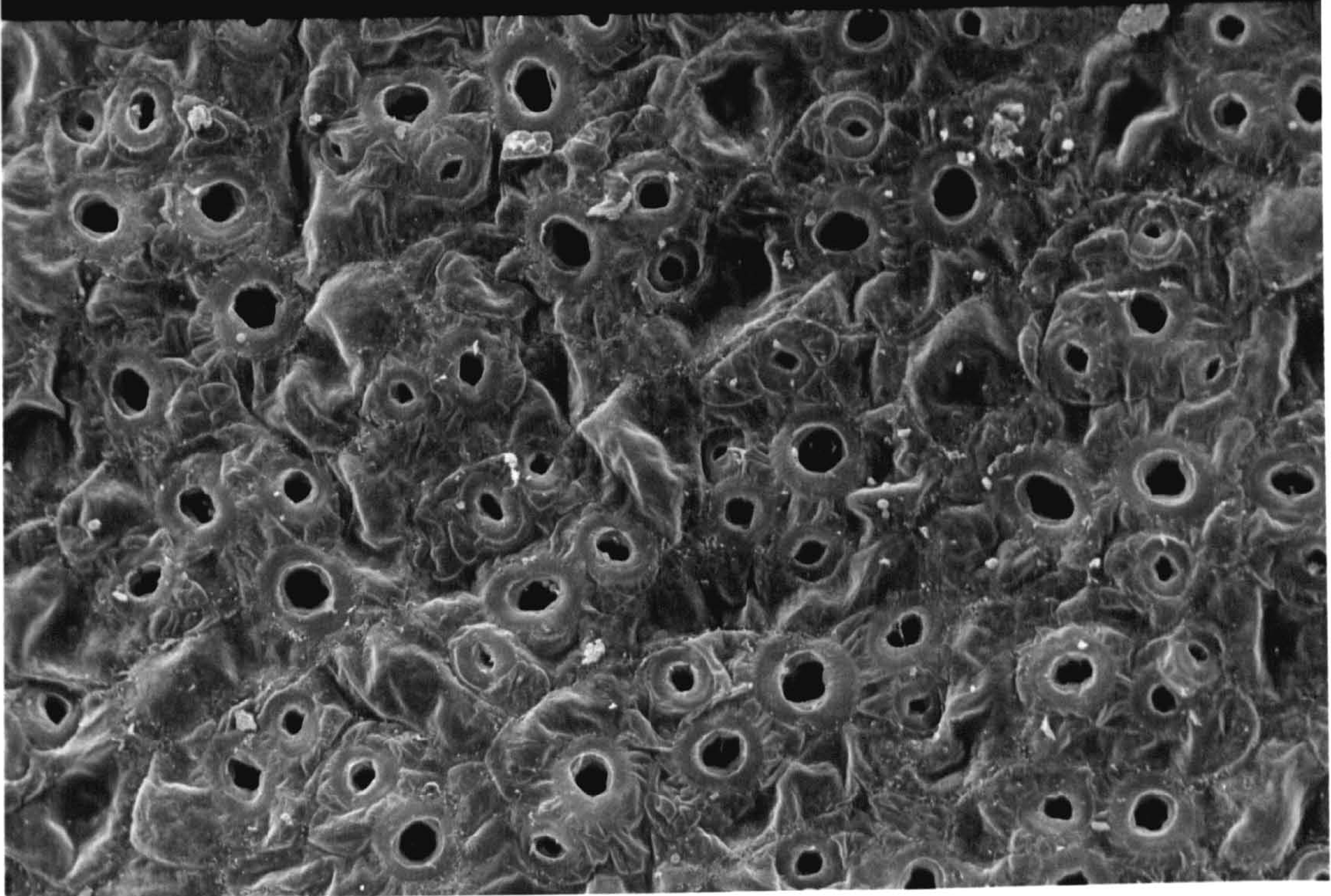
## PLATE : 6.11

**Cauliflower :** scanning electron micrographs of lower epidermis of 3rd or 4th leaf from apex of 15 days old plantlets grown in continuous light (PAR = 150 μmol $m^{-2}s^{-1}$) and under different types of ventilation as follows:

(above) sealed condition (silicone rubber bung); note larg stomata, densely arranged and many gaping widely open .

(below) diffusive ventilation (polypropylene disc), note large stomata, less densely arranged than above and some gaping widely open .

In both of these treatments the stomata appeared to be in 'elevated' positions; some, perhaps immature ones were quite small and relatively closed. Also the epidermal cells, (other than the guard cells), were less highly organised than in Plate 6.12, where the ventilation of the plantlets was more efficient.


100 µm

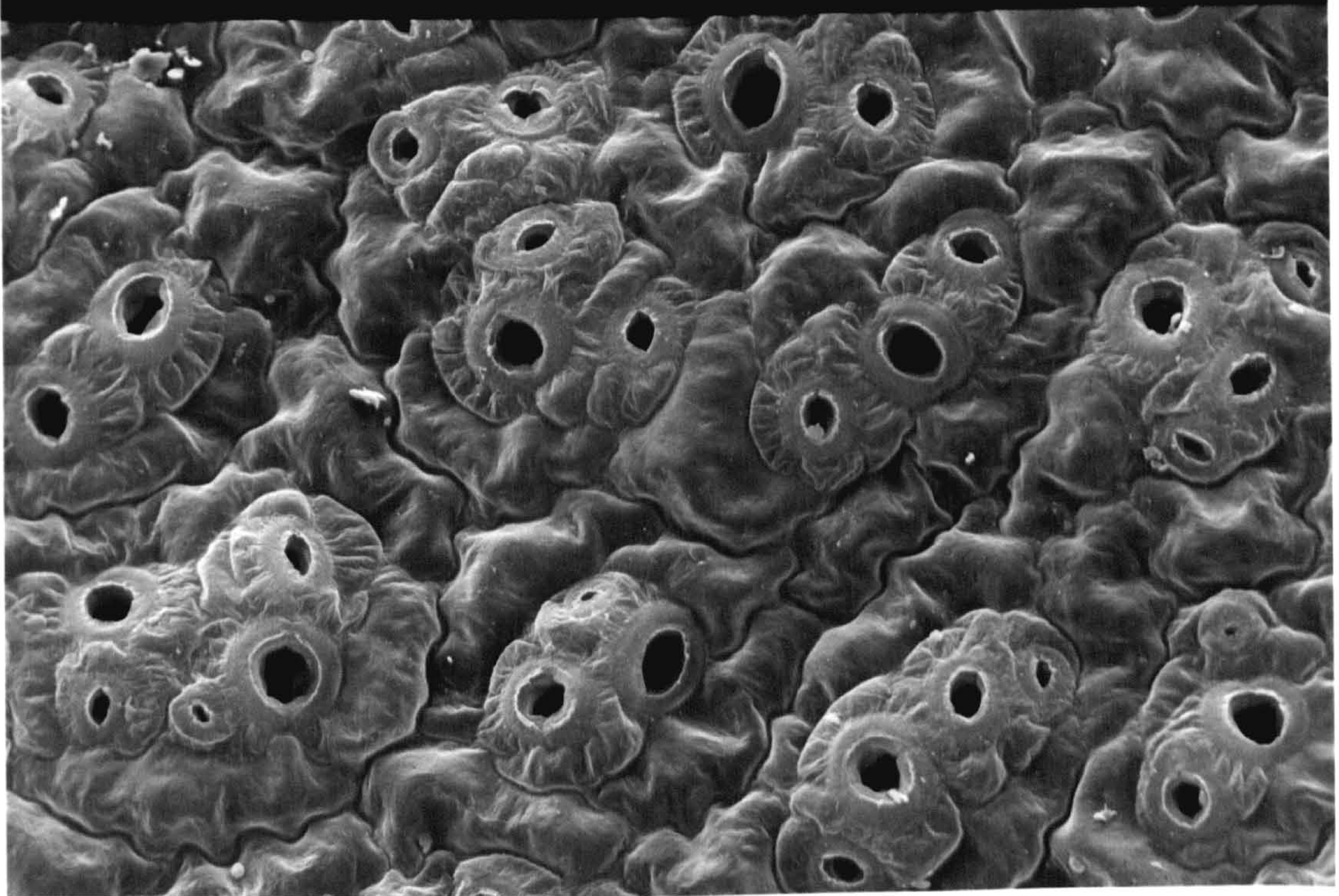

100 µm

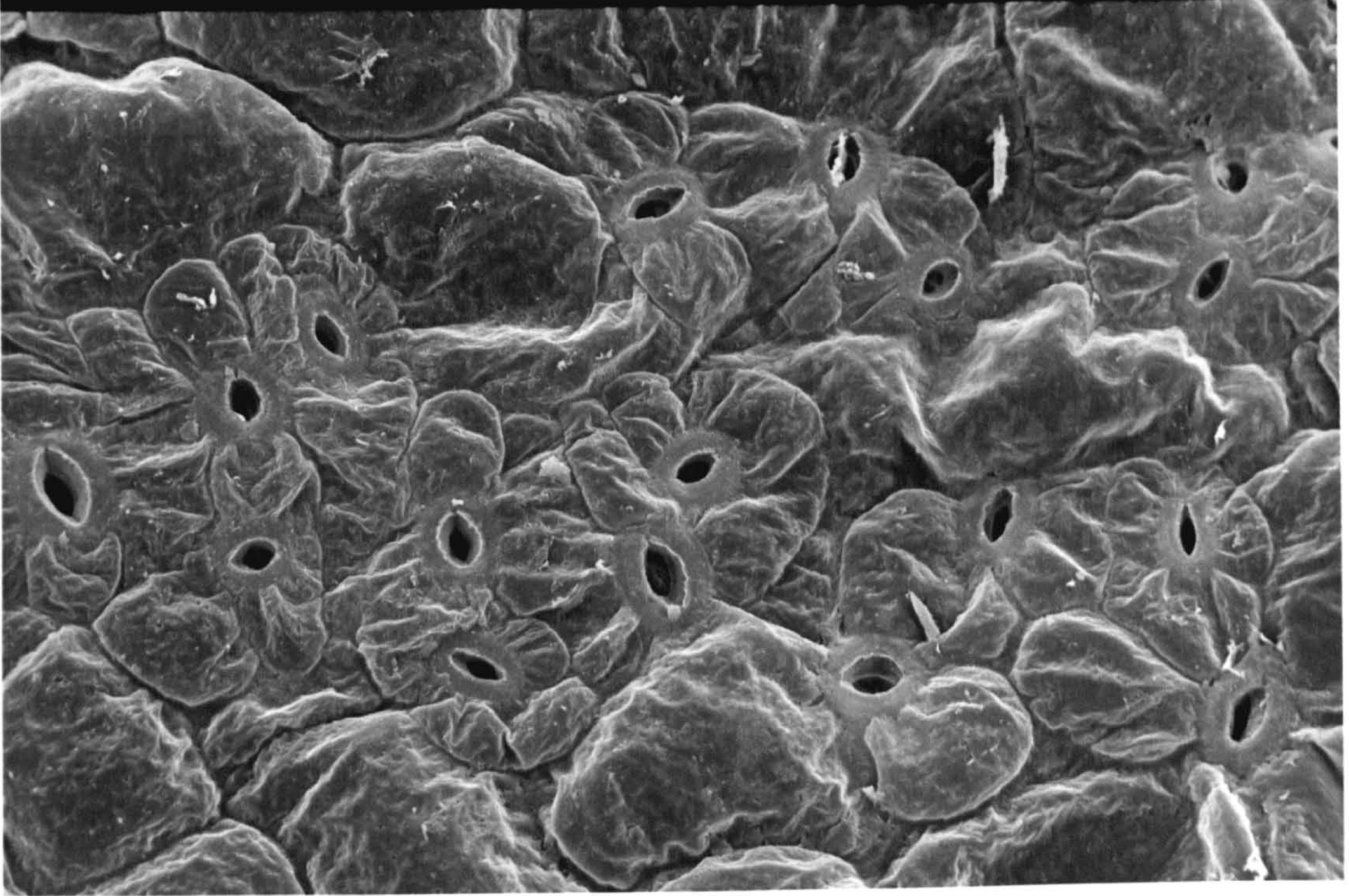
**PLATE : 6.12**

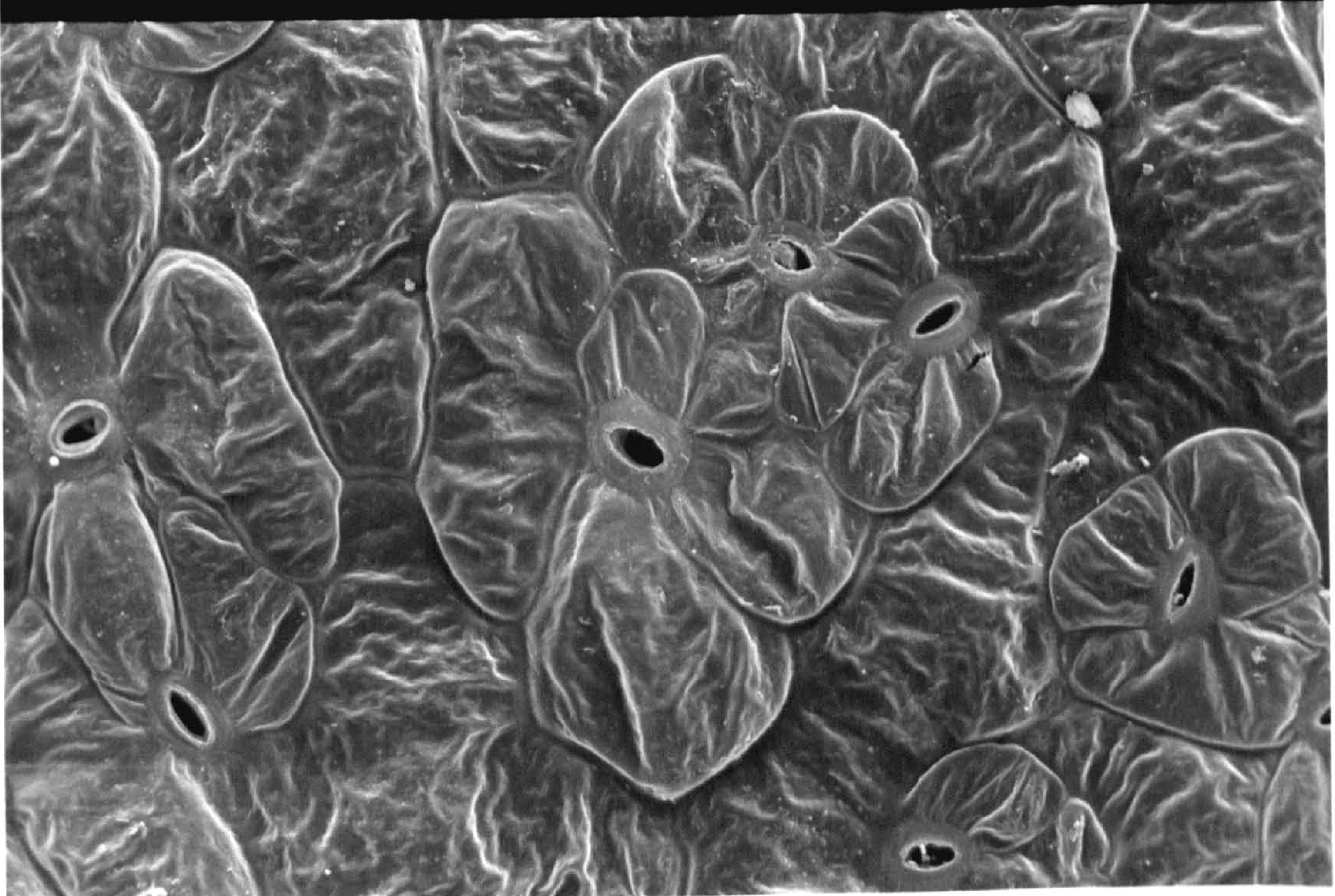
**Cauliflower:** scanning electron micrographs of lower epidermis of 3rd or 4th leaf from apex of 15 days old plantlets grown in continuous light (PAR = 150 $\mu mol\ m^{-2}s^{-1}$) and under different types of ventilation as follows:

(above) slow flow ventilation (flow rate = 1.0 - 1.5 $cm^3\ min^{-1}$); note that stomata were smaller and less densely arranged than in Plate 6.11 and stomatal pores were narrower in width.

(below) fast flow ventilation (flow rate = 5.0 $cm^3\ min^{-1}$); note that smaller stomata, less densely arranged than in Plate 6.11 and also stomatal pores were narrower in width.

In both of these treatments the stomata appeared to be slightly sunken; also the epidermal cells, (other than the guard cells), were far more highly organised than in Plate 6.11, where the ventilation of the plantlets was less efficient.


100 µm


100 µm

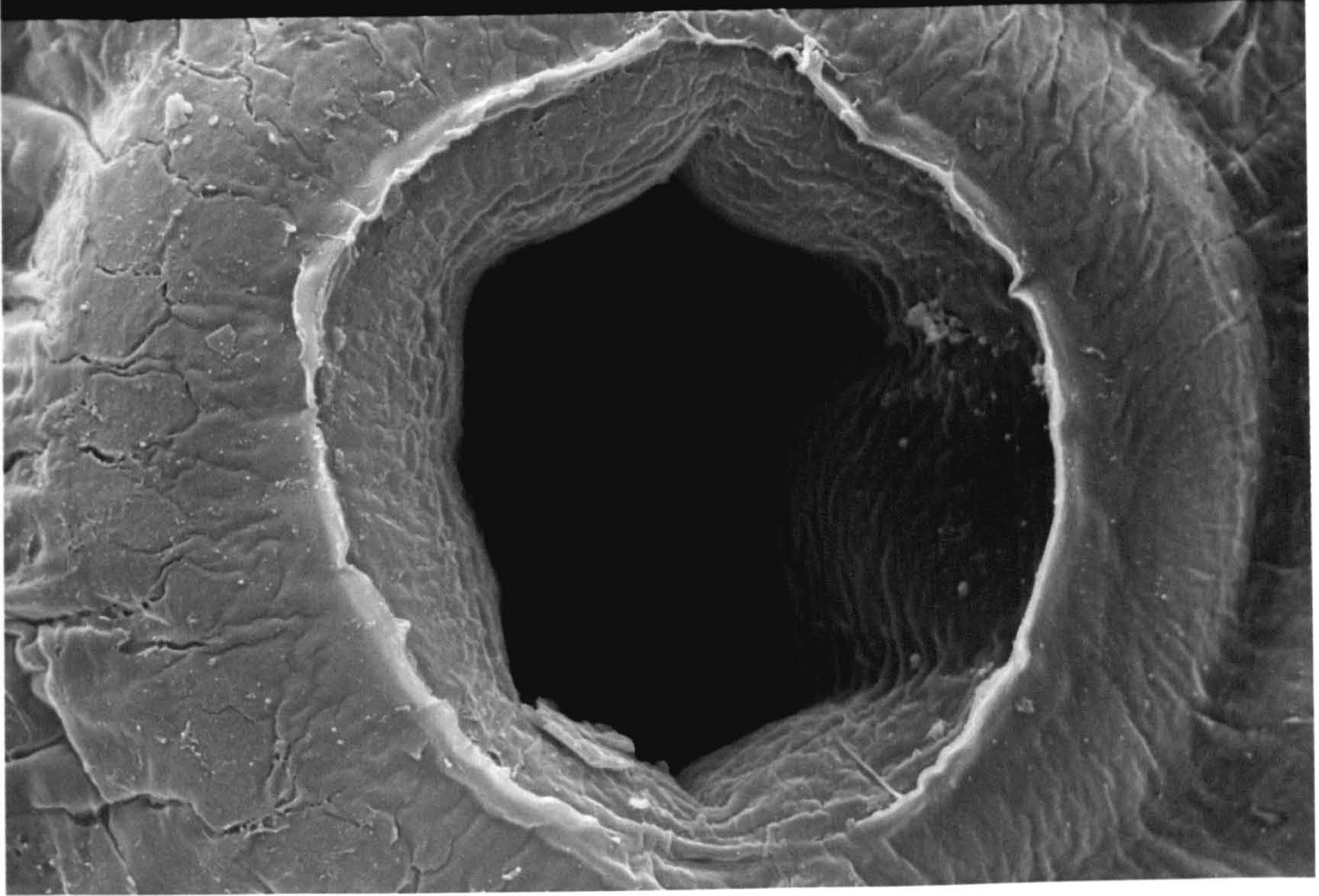
## PLATE : 6.13

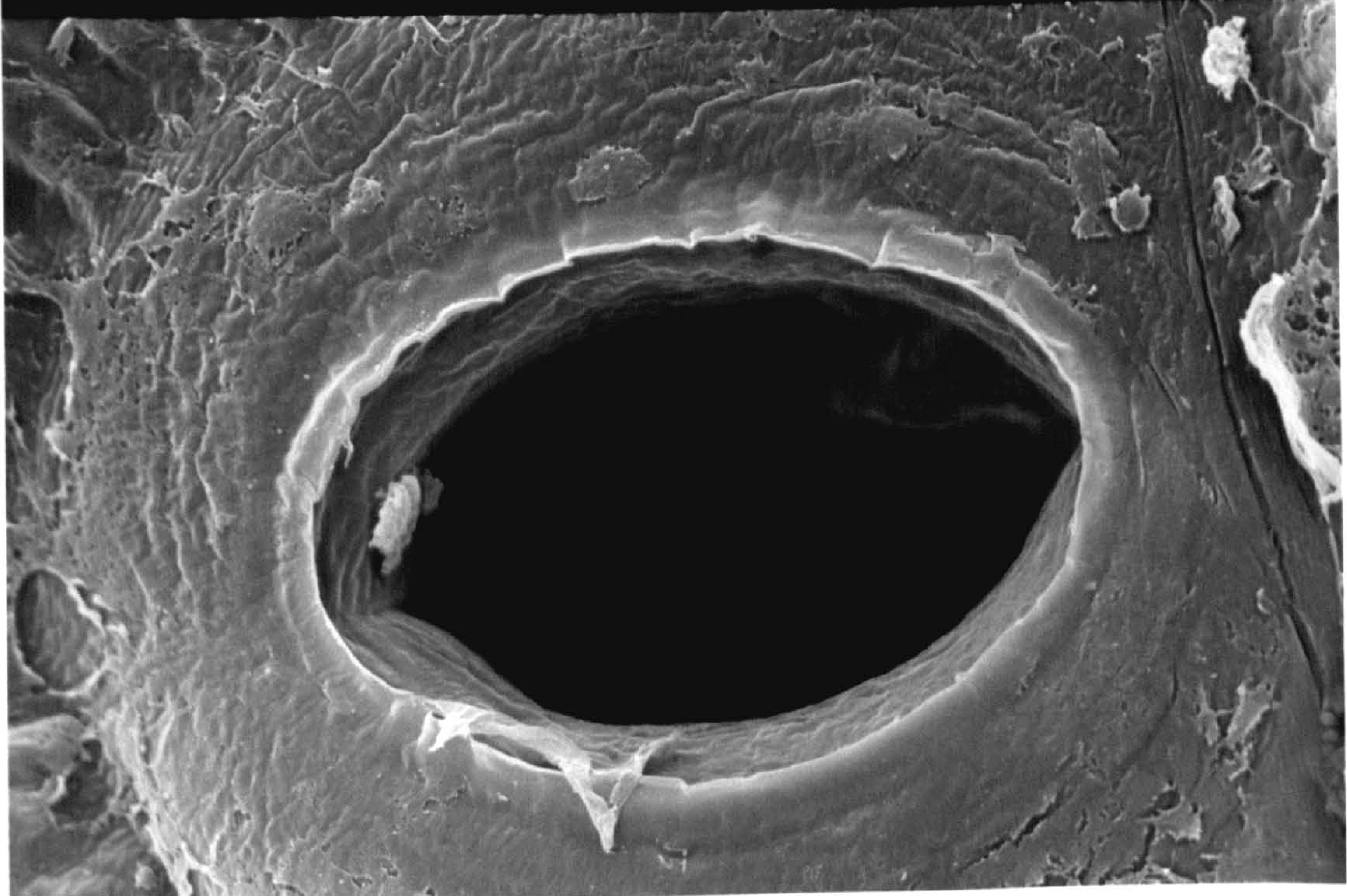
**Cauliflower :** scanning electron micrographs showing enlarged stomata from Plate 6.11. (Plants grown in continuous light).

(above) sealed condition (silicone rubber bung)

(below) diffusive ventilation (with a polypropylene disc)

Note large size of stomata and pores compared to Plate 6.14.


10.0µm


10.0µm

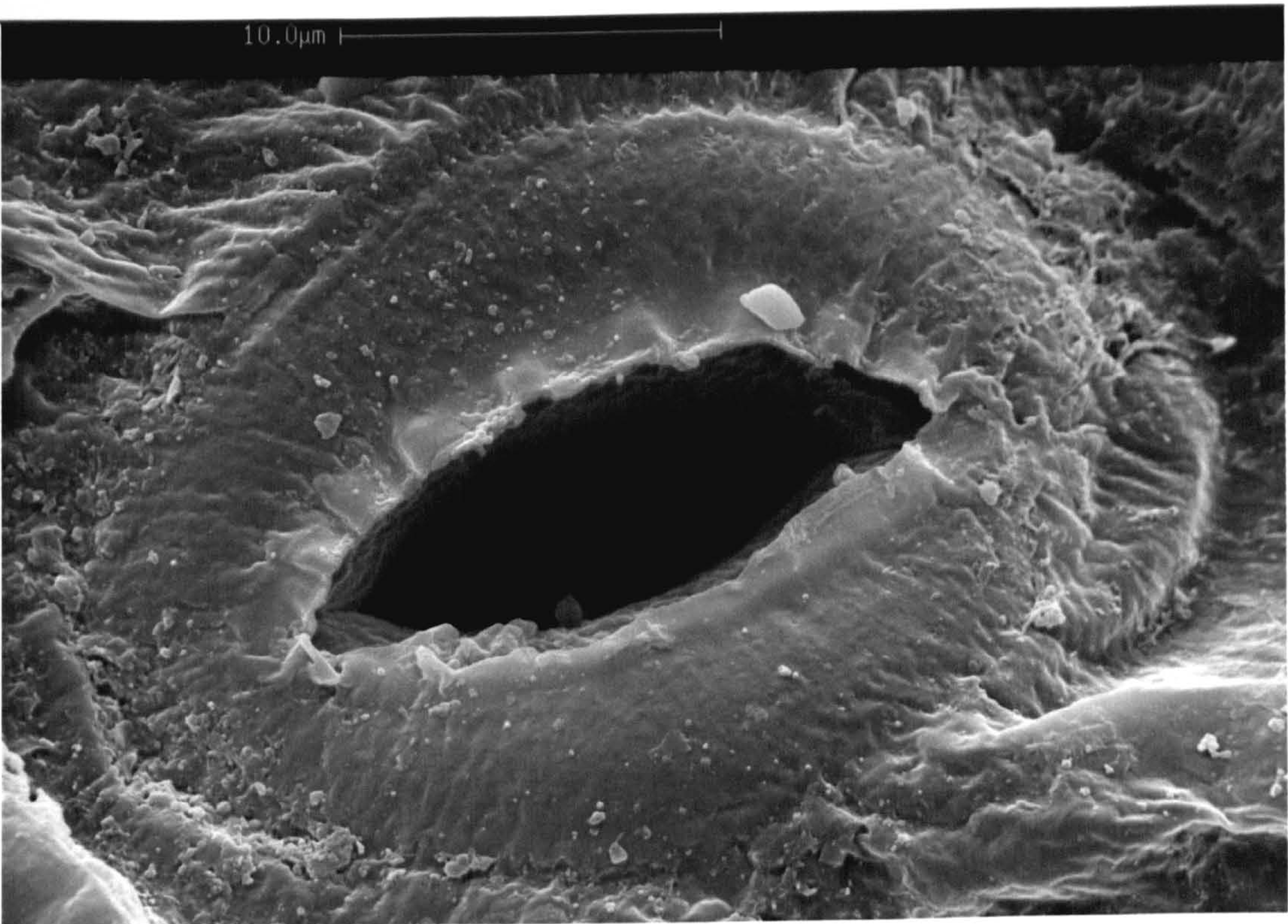
## PLATE : 6.14

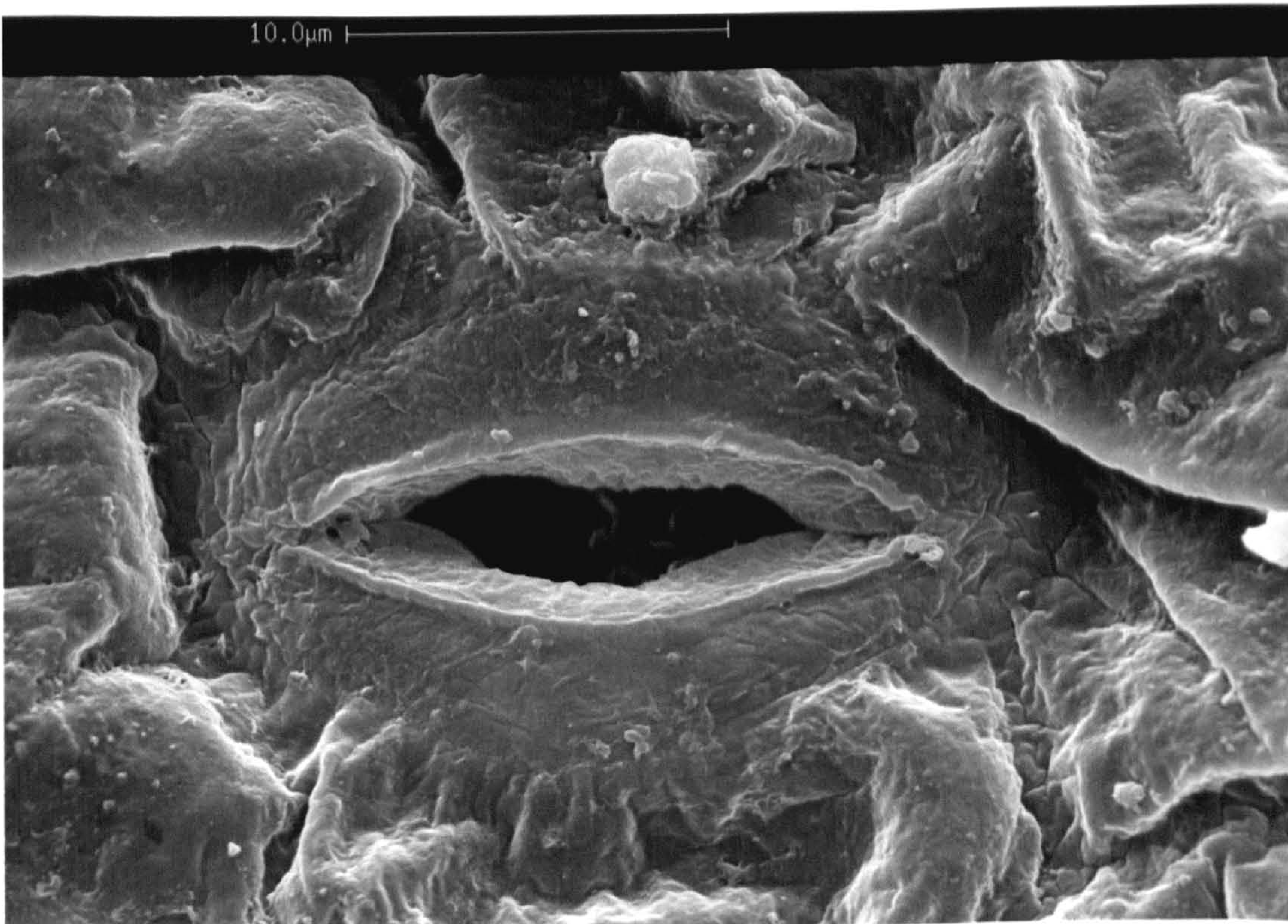
**Cauliflower :** scanning electron micrographs showing enlarged stomata from Plate 6.12. (Plants grown in continuous light).

(above) slow flow ventilation (1.0 - 1.5 $cm^3$ $min^{-1}$).

(below) fast flow ventilation (5.0 $cm^3$ $min^{-1}$).

Note smaller stomata and narrower pores compared to Plate 6.13.


10.0µm


10.0µm

## 6.4. FINAL COMMENTS

It should be stressed that the anatomical investigation described in this chapter was a preliminary one. This was because the study was undertaken during the final stage of the project, when time was short. To draw definite conclusions about the open or closed state of the stomata under different conditions of ventilation, would require studies using a porometer. Also, ideally, more replication would be necessary in the methods in general.

However, the study has pointed the way to further investigations and has indicated that poor ventilation, (sealed and diffusive treatments), within culture vessels can lead to the development of anatomical features which could prevent or reduce the plant's ability to acclimatise. These include an absence or reduction in leaf epicuticular and cuticular waxes in cauliflower, and in tobacco a reduction in lengths of epidermal hairs, and in both species, permanently widely-open stomata which do not respond normally by closing in the dark or under conditions of low RH, and which are in abnormally exposed positions on the leaf. It would be reasonable to conclude that all these features would lead to abnormally and inherently high rates of transpiration, which could not be controlled during a period of acclimatisation into conditions of lower RH, and which could jeopardise the plant's chances of survival. There are indications that these anatomical and physiological modifications may be induced in culture vessels with poor ventilation because of very high humidity, and/or low $CO_2$ concentrations and/or high concentrations of ethylene.

On the other hand, with forced ventilation and in the *in vivo* condition the stomata behaved more normally by closing in the dark and when exposed to low RH.

Finally, the study indicates that these adverse effects can be prevented or reduced by the introduction of forced ventilation within the culture vessels.

# CHAPTER VII

## EFFECTS OF DIFFERENT TYPES OF VENTILATION ON GROWTH, LEAF AND FLOWER-BUD ABSCISSION, PHOTOSYNTHETIC RATES AND ETHYLENE AND $CO_2$ CONCENTRATIONS IN *ANNONA* CULTURES

### 7.1. INTRODUCTION

*Annona*, *Rollinia* and *Asimina* are members of the Annonaceae family which produce edible fruits (George and Nissen 1993). The genus *Annona* includes about 100 species, of which the main commercial ones are the sugar apple comprising (*Annona squamosa* L.), the cherimoya (*Annona cherimola* Mill.), and the hybrid, atemoya (*Annona cherimola* Mill. X *Annona squamosa* L.), and the soursop (*Annona muricata*) (Rasai, George and Kantharajah 1995). The first three fruits are generally known also as 'custard apples' (Brown *et al.* 1988), while *A. muricata* is sometimes called the 'prickly apple'.

The custard apple or sugar apple (*Annona squamosa* L.), a tropical fruit tree native to South America, the West Indies and now cultivated also in India, has the potential to become a major horticultural crop (George and Nissen 1987). Prickly custard apple (*Annona muricata*) is an evergreen tree, native to tropical America, and also widely cultivated in the tropics for its fruit (Bejoy and Harihran 1992). Cherimoya is native to the subtropical highlands of Peru and Ecuador and is commercially grown in Chile, Spain, California and New Zealand (George and Nissen 1993). Atemoya is grown commercially in Florida (USA) and Australia (George, Nissen and Brown 1987).

Currently the *Annona* spp. are propagated through grafting and budding (Rasai, George and Kantharajah 1995), since for almost all of the species of *Annona* the clonal propagation by cutting or air layering has not been very successful (Rasai, George and Kantharajah 1995). Seedling rootstocks used for vegetative propagation are highly variable in vigour and disease resistance and consequently scion growth and productivity are also variable (George and Nissen 1987). Seed germination of *Annona squamosa* species in nature is only about 30 - 40%. Tree improvement in *Annona muricata* becomes difficult because no success has been achieved so far in crossing this species with other *Annona* species (Samson 1986). *A. muricata* has normally been propagated

from seeds because vegetative propagation through conventional methods is very slow and costly. Alternatively, for the rapid multiplication of new genotypes and for the elimination of viral and disease infection of horticultural species, tissue culture is an important tool (Frey 1981). However, there have been some problems concerning *in vitro* propagation of Annonas (Rasai, George and Kantharajah 1995). These include (i) browning of media (Nair, Gupta and Mascarenhas 1983; Rasai, Kantharajah and Dodd 1994), (ii) very low success rate of rooting (Jordan 1988) and shoot multiplication, (iii) a high rate of ethylene-induced leaf abscission (Rasai, Kantharajah and Dodd 1994; Lemos and Blake 1994; Armstrong *et al.* 1996) and (iv) low rate of bud opening (Lemos and Blake 1994).

The present investigation was instituted in the hope of overcoming some of these problems. To this end a system for the micropropagation of *Annona squamosa* and *Annona muricata* using nodal explants of greenhouse grown plants was developed using forced ventilation of the culture vessels. This chapter describes the propagation methods and compares effects of sealed-, diffusive- and forced-ventilation on micropropagation, leaf and flower-bud abscission, growth and development and photosynthetic rate etc. on the two species. The effects of ventilation on the concentrations of ethylene and $CO_2$ in the culture head-space and the influence of various hormones on shoot regeneration and flower-bud initiation are also described.

## 7.2. MATERIALS AND METHODS

### 7.2.1. Plant materials and sterilization

Experimental material originated as nodal explants taken from 2 to 3 years old greenhouse-grown plants *Annona squamosa* and *A. muricata*. Explants with 4 to 5 nodes were cut from young shoots, and to remove dirt were brushed lightly and carefully so as not to break or damage the axillary buds. Nodal segments were separated, washed under running tap-water and surface-sterilized by dipping into sodium hypochlorite solution (2% w:v) for 5 min. They were then rinsed three times with sterile water, cut into 12 -15 mm lengths and finally inoculated on to agar media in culture vessels.

Unless stated otherwise, the establishment conditions, culture vessel size and volume, media preparation, methods of measuring ethylene and $CO_2$ concentration, chlorophyll and carotenoid contents, photosynthetic rates etc., were as described in Chapter IV The basal medium used was MS (Murashige and Skoog 1962).

### 7.2.2. Experiments

#### 7.2.2.1. Micropropagation

To discover a suitable medium for shoot induction, surface-sterilized nodal segments of both *A. squamosa* and *A. muricata* were inoculated into culture vessels containing media supplemented with different combinations and concentrations of BAP and NAA (Table 7.01 and 7.02). Each vessel was capped with a polypropylene disc and only one explant was inoculated per vessel. Cultures were incubated at 25°C under a continuous light flux of *ca.* 80 $\mu$mol $m^{-2}$ $s^{-1}$. Harvesting was after 7 weeks; measurements included % of tubes with shoots or buds, number of buds per explant % of elongated shoots, lengths of elongated shoots.

#### 7.2.2.2. Effects of different types of ventilation on multiple shoot induction

Surfaced-sterilized nodal segments of both species were inoculated onto MS medium supplemented with BAP (1.5 mg $l^{-1}$) + CH (1.0 g $l^{-1}$) for *A. squamosa* and BAP (1.0 mg $l^{-1}$) + NAA (0.1 mg $l^{-1}$) for *A. muricata* (the species had shown the best responses in

these combinations). Each vessel was capped with either (a) a silicone rubber bung (sealed control),(b) a silicone rubber bung and injected with 10 ml of 22 $\mu l\ l^{-1}$ ethylene gas, (c) a disc of polypropylene membrane to allow diffusive ventilation, (d) a FF-ventilation apparatus (flow rate = 5.0 $cm^3\ min^{-1}$ produced by having a larger outflow membrane and closer evaporating surface than in Chapters IV and V) for forced ventilation, or (e) grown under diffusive ventilation and then transferred to forced ventilation.

Ten replicates were prepared for each treatment and the cultures were incubated in growth room conditions. On the 35th day the ethylene concentrations were measured in the vessels. The dates of bud initiation were noted. Harvesting was after 35 days; measurements included number of buds, % of elongated shoots, length of shoots, number of leaves per shoot, leaf area per shoot and number of nodes per shoot.

#### 7.2.2.3. Effects of different types of ventilation on leaf abscission

For this experiment, 14-day-old *A. squamosa* and *A. muricata* plantlets cultured in MS medium supplemented with BAP (2.0 mg $l^{-1}$) and aerated by forced ventilation (5.0 $cm^3\ min^{-1}$) were used. Only 1-2 main shoots were allowed to grow and the rest were chopped off. The plantlets were then sub-cultured into fresh medium and each vessel was capped with either (a) a silicone rubber bung (sealed control), (b) a silicone rubber bung and then injected with 10 ml of 22 $\mu l\ l^{-1}$ ethylene gas, (c) a disc of polypropylene membrane for diffusive ventilation, (d) a FF-ventilation apparatus (flow rate = 5.0 $cm^3\ min^{-1}$) for forced ventilation. Ten replicates were prepared for each treatment. The percentage leaf abscission was measured from days 1 - 45. Ethylene concentrations were measured on day 45.

#### 7.2.2.4. Flower-bud development

Nodal segments of *A. muricata* were inoculated onto MS medium supplemented with different combinations and concentrations of BAP and CH. Only one explant was inoculated per vessel and the vessels were capped with a FF-ventilation apparatus.

Cultures were incubated for 60 days under the growth room conditions described earlier. Dates of flower bud initiation were noted. Harvesting was on day 45 and measurements included % of tubes with flower-bud development, numbers of flower-buds and fresh weight of each flower bud.

#### 7.2.2.5. Effects of different types of ventilation on flower-bud development and abscission

Annona muricata plantlets (20 days old) cultured in MS + BAP (2.0 mg $l^{-1}$) + CH (1.0 g $l^{-1}$) medium and grown under forced ventilation were used as experimental material. Plantlets were subcultured into fresh medium and, to provide different ventilation treatments, the vessels were capped and grown as described in 7.2.4. Initially five representative plants were harvested and the number of flower-buds and their weights were recorded. For the "treatment plants" the number of new flower-buds developed and abscissions and the fresh weights of dropped flower-buds were recorded throughout the experiment. Harvesting was at 21 days: ethylene concentrations were measured and fresh weights of flower-buds recorded the at the end of the experiment Five replicates were prepared for each treatment

#### 7.2.2.6. Effects of different types of ventilation on growth, photosynthesis, and chlorophyll and carotenoid contents

Nodal segments of both *Annona squamosa* and *A. muricata* were inoculated onto MS + BAP (2.0 mg $l^{-1}$) and, to provide the different ventilation treatments, the vessels were capped and grown as described in Section 7.2.4. Initially the fresh weights of the cuttings were taken. Cultures were incubated in growth room conditions. The number of replicates was 10 for each treatment. The photosynthetic rates and chlorophyll and carotenoid contents were determined after 45 days of growth. Harvesting was after 45 days and the following measurements made: number, fresh weight and area of leaves (including dropped leaves), stem fresh weights, lengths, number of new nodes, total number of buds and number of opened buds.

### 7.2.2.7. Effects of different ventilation types on the gaseous composition of the head-space of the culture vessels

Plantlets of both *A. squamosa and A. muricata*, 35 days old and with 1-3 shoots, having been grown under fast forced ventilation were subcultured to a fresh medium in new vessels. The effects of the plantlets in each case on the gas composition of the head-spaces were then investigated under the various ventilation treatments. Five replicates for each treatment were prepared for each species. Each vessel (+plantlet) was subjected in turn to the three types of ventilation viz. (i) forced flow (FF-apparatus), (ii) diffusive (polypropylene disc capping), and (iii) closed (sealed using silicone rubber bung). Cultures were incubated at 25°C and at a PAR of 80 µmol $m^{-1}$ $s^{-1}$ with an 18 h photoperiod; there were 5 replicates per treatment.

For both species $CO_2$ concentrations in the culture vessels were measured at intervals throughout the day and night. The measurement procedure has been described in Chapter IV.

Also for both species ethylene concentrations in the light were measured in each culture vessel at intervals during the first 48 hours.

In an extra experiments on *A. squamosa*, 5 replicates, similarly prepared as above, were sampled for ethylene during 29.75 hours in the light. The vessels were then uncapped and flushed with sterile air and then recapped. Ethylene concentrations were again measured during the following 29.75 hours, and the procedure was repeated.

## 7.3. RESULTS AND DISCUSSION

### 7.3.1. Micropropagation

***Annona squamosa*** : To regenerate shoots from nodal segments of mature plants without an intervening callus phase, up to 18 different hormonal treatments were given of which only 5 stimulated adventitious shoot buds (Table : 7.01). The best response was recorded in **medium 1** : MS + BAP (1.5 mg $l^{-1}$) + CH (1.0 g $l^{-1}$). Within 7-10 days one or two shoots developed from the leaf axil, and after 3 weeks of culture new lateral buds were initiated in this medium. With the passage of time the number of buds increased and reached a mean of *ca.* 70 within 5 weeks. At the end of the experiment, however, only 39% of shoots had elongated. After 8-9 weeks, longer shoots ($\geq$ 1 mm) were detached and transferred onto the rooting medium (data not shown). The original explants were then subcultured to fresh medium where elongation of old shoots and initiation of new shoots took place. It should be mentioned that from a single explant it was possible to regenerate more than 100 new shoots. Regenerated shoots from the explant were also used as a source of secondary explants and these exhibited a similar response. In this way the shoot multiplication cycle can be repeated indefinitely.

**Table 7.01.** **Effects of different hormonal treatments on regeneration of shoots from nodal segments of mature *Annona squamosa* after 7 weeks of culture.**

| * Media used | % of tubes with shoots /buds developed | No. of buds developed per explant | % shoots elongated | †Length of elongated shoots (mm) |
|---|---|---|---|---|
| Medium - 1 | 97.6 | 43.2±9.6 | 39.9±5.3 | 0.93±0.05 |
| Medium - 2 | 83.4 | 24.3±4.2 | 40.4±3.1 | 0.81±0.08 |
| Medium - 3 | 51.0 | 19.1±5.7 | 42.2±3.5 | 0.92±0.11 |
| Medium - 4 | 33.1 | 15.2±1.9 | 34.8±3.2 | 1.01±0.07 |
| Medium - 5 | 28.2 | 3.7±0.7 | 89.2±8.1 | 1.27±0.15 |

***Medium - 1** : MS + BAP (1.5 $mgl^{-1}$) + CH (1.0 $gl^{-1}$); **Medium - 2** : MS + BAP (2.0 $mgl^{-1}$) + CH (1.0 $gl^{-1}$); **Medium - 3** : MS + BAP (2.0 $mgl^{-1}$); **Medium - 4** : MS + BAP (2.5 $mgl^{-1}$) + NAA (0.1 $mgl^{-1}$) + CH (1.0 g $l^{-1}$); **Medium - 5** : MS + BAP (2.5 $mgl^{-1}$) + NAA (0.5 $mgl^{-1}$)

*60 $cm^3$ vessels, each containing one cutting, were capped with polypropylene disc. Cultures grown at *ca.* 25°C in continuous light ; PAR = 80 µmol $m^{-2}s^{-1}$. Each value represents a mean ± SE of 5 replicates.

†mean maximum length (3 - 5 best shoots from each tube).

Medium 2 [MS + BAP (2.0 mg $l^{-1}$) + CH (1.0 g $l^{-1}$)], and medium 3 [MS + BAP (2.0 mg $l^{-1}$)], also showed noticeable numbers of shoots (Table 7.01), but when compared with those of medium 1, the number and percentage of shoots in each tube was lower. However, percentages of elongated shoots were slightly higher in these treatments. Medium 4 with a mean of only 15 shoots per explant was only one third of that of medium 1. In the case of medium 5, however, the mean number of shoots was only 3.7 (i.e. only 0.085X) that of in medium 1), and this was probably a function of the NAA addition.

***Annona muricata*:** Among the treatments the five best responses are represented in Table 7.02. In medium 1, the mean number of buds was approx. five out of which only 46% elongated with an average shoot length of *ca.* 11 mm by the end of the experiment. In medium 2 the number of buds was again 5.0 but this was reduced significantly when NAA was not added in the medium (mediums 3, 4 and 5), but here the growth was better with at least double length of shoots and significantly higher numbers of elongated shoots (= 75%). It should be mentioned that, in general, leaf abscission is a major problem in *A. muricata* (described later) and when NAA was added in the medium abscission was increased (data not shown).

**Table 7.02. Effects of different treatments on regeneration of shoots from nodal segments of mature *Annona muricata* after 7 weeks of culture.**

| * Media used | % of tubes with shoots /buds developed | No. of buds developed per explant | % shoots elongated | †Length of elongated shoots (mm) |
|---|---|---|---|---|
| Medium - 1 | 79.1 | 5.2±1.6 | 46.3±3.7 | 11.3±0.5 |
| Medium - 2 | 73.7 | 5.0±4.2 | 39.9±3.4 | 11.9±0.8 |
| Medium - 3 | 89.0 | 3.1± 0.7 | 75.0±7.1 | 21.0± 3.1 |
| Medium - 4 | 83.0 | 2.3± 0.5 | 73.0±6.9 | 19.3±4.1 |
| Medium - 5 | 75.0 | 1.7± 0.3 | 59.1±4.6 | 18.5±2.5 |

* **Medium - 1** : MS + BAP (1.0 mg$l^{-1}$) + NAA (0.1 mg$l^{-1}$); **Medium - 2** : MS + BAP (2.0 mg$l^{-1}$) NAA (0.1 mg$l^{-1}$); **Medium - 3** : MS + BAP (1.0 mg$l^{-1}$); **Medium - 4** : MS + BAP (2.0 mg$l^{-1}$); **Medium - 5** : MS + BAP (2.5 mg$l^{-1}$) + NAA (0.5 mg$l^{-1}$). *60 $cm^3$ vessels, each containing one cutting, were capped with polypropylene disc. Cultures grown at *ca.* 25°C in continuous light; PAR = 80 µmol $m^{-2}s^{-1}$. Each value represents a mean ± SE of 5 replicates. †mean maximum length.

Similar results were described by Nair *et al.* 1984 where in the presence of auxin (NAA, IAA and IBA) in the medium, the leaves of *A. squamosa* were dropped. It is well known that additions of auxin can promote ethylene production in plants by stimulating the biosynthesis of ACC (Yang and Hoffman 1984).

### 7.3.2. Effects of different types of ventilation on multiple shoot induction

Shoot length was sometimes difficult to measure, but was possible using digital electronic callipers.

***Annona squamosa*** : The results are summarised in Table 7.03. Among the treatments, bud initiation was observed within 7 days in the sealed vessels but the number was only 2.3 (data not shown), and with the passage of time these buds became brown in colour with some necrosis and ultimately died. Ethylene concentrations in the head-space of these sealed culture vessels after 50 days was 0.76 µl $l^{-1}$ (Fig. 7.01). Explants cultured in an atmosphere with added ethylene showed no bud or shoot development.

**Table 7.03.** **Effects of different types of ventilation on multiple shoot induction of *Annona squamosa* after 7 weeks of culture.**

| Characteristics | Sealed with silicone rubber bung | ❖ Sealed + injected ethylene | Diffusive ventilation (polypropylene disc) | Forced ventilation (flow rate = 5.0 $cm^3$) | Grown under diffusive vent. and than transferred to forced ventilation |
|---|---|---|---|---|---|
| Days to bud initiation | 5 -7 | No buds developed | 7 -10 | 12 - 15 | --- |
| Number of buds per plant | --- | --- | 48.7±6.7 | 25.5±1.3 | 39.2 ± 4.9 |
| % of buds elongated | --- | --- | 38.7 | 74.1 | 69.2 |
| †Length of young shoots (mm) | --- | --- | 0.5±0.1 | 1.67±0.2 | 1.2±0.2 |
| No. of leaves per young shoot | --- | --- | 1.4±0.2 | 5.5±0.6 | 5.2±0.4 |
| Leaf area per young shoot ($cm^2$) | --- | --- | 0.5±0.1 | 2.0±0.3 | 1.2±0.3 |
| No. of nodes per young shoot | --- | --- | 1.1±0.3 | 3.0±0.7 | 1.6±0.5 |

†mean maximum length of 5 shoots from each tube. ❖10 $cm^3$ of 23 µl $l^{-1}$ ethylene in air was injected. *60 $cm^3$ vessels, each containing one cutting. Cultures grown at *ca.* 25°C in continuous light; PAR = 80 µmol $m^{-2}s^{-1}$. Each value represents a mean ± SE of 5 replicates. Elongated buds become young shoots.

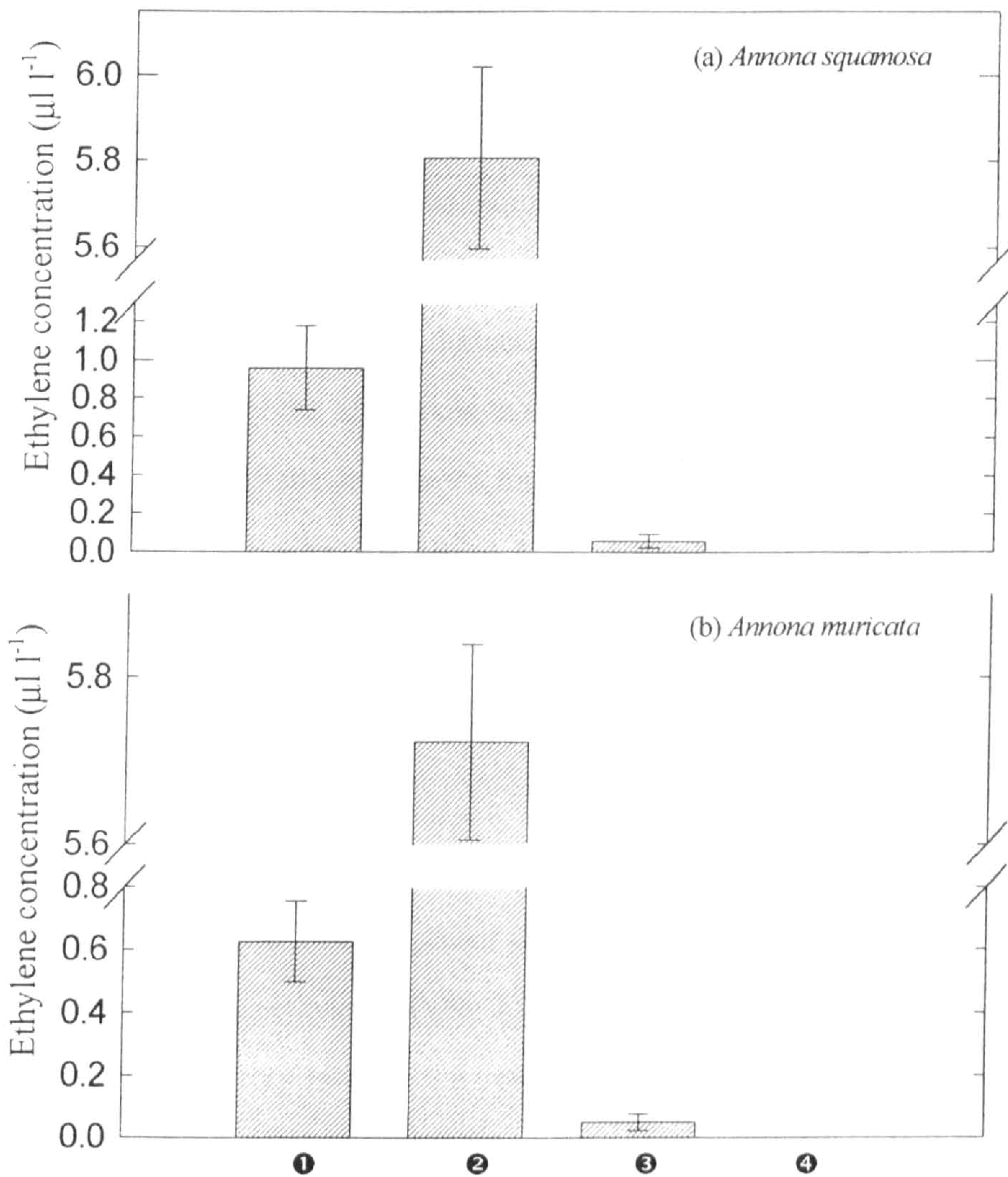



**Fig.7.01.** Effects of different types of 'capping' on ethylene concentrations in the head-space above 50 days old (a) *A. squamosa* L. and (b) *A. muricata* L. cultures in 60 $cm^3$ vessels; cultures were grown under continuous light at 80 μmol $m^{-2}$ $s^{-1}$ (PAR), 25°C and 21% RH. Each symbol represents a mean ± SE of 5 replicates. ❶ = sealed with silicone rubber bung; ❷ = sealed with silicone rubber bung and 10 $cm^3$ ethylene (23 μl $l^{-1}$ ethylene in air) was injected in each vessel immediately after sealing; ❸ = capped with polypropylene disc (diffusive ventilation) and ❹ = capped with fast forced ventilation apparatus (flow rate = 5.0 $cm^3$ $min^{-1}$).

With continuous diffusive ventilation bud initiation took place within 7 - 10 days, compared to 12 - 15 days with continuous forced ventilation. Although the most buds were recorded with the former treatment, with forced ventilation 74% of buds had elongated after 7 weeks (Plate 7.01c), compared to only 39% with diffusive ventilation. In the latter case few buds remained unopened, and some ultimately died (Plate 7.01b). Similarly all other parameters were increased by forced ventilation, with shoot length 2X, number and area of leaves 4X and number of nodes 3X those from the diffusive treatment.

For plants transferred from diffusive into forced ventilation (Plate 7.02b), apart from the number of buds, all parameters were intermediate between those for the continuous diffusive and the continuous forced ventilation treatments. This showed that forced ventilation, even when applied at a late stage (after 25 days) can still improve growth and development, particularly here in the case of the percentages of elongated shoots and numbers of leaves, which were close to those from the continuous forced ventilation treatment.

***Annona muricata***: This species, in contrast to *A. squamosa*, did not show any sign whatsoever of bud or shoot development in the sealed containers either in the presence or absence of exogenous ethylene (Table 7.04).

The pattern of responses to the ventilation treatments was similar to that for *A. squamosa*, with all growth parameters, apart from the number of buds, being increased by forced as opposed to diffusive ventilation. However, in *A. muricata* the numbers of buds were much smaller ($\leq$ 0.16X those of *A. squamosa*), but the lengths of shoots were greater (>10X those for *A squamosa*). Here again the late application of forced ventilation had beneficial effects, particularly in the cases of percentage of elongated shoots and numbers of leaves per shoot.

Ethylene concentrations measured at the end of the experiments were similar in both *A. squamosa* and *A muricata* (Fig. 7.01) with respectively 0.95 - 0.62 $\mu l\ l^{-1}$ in

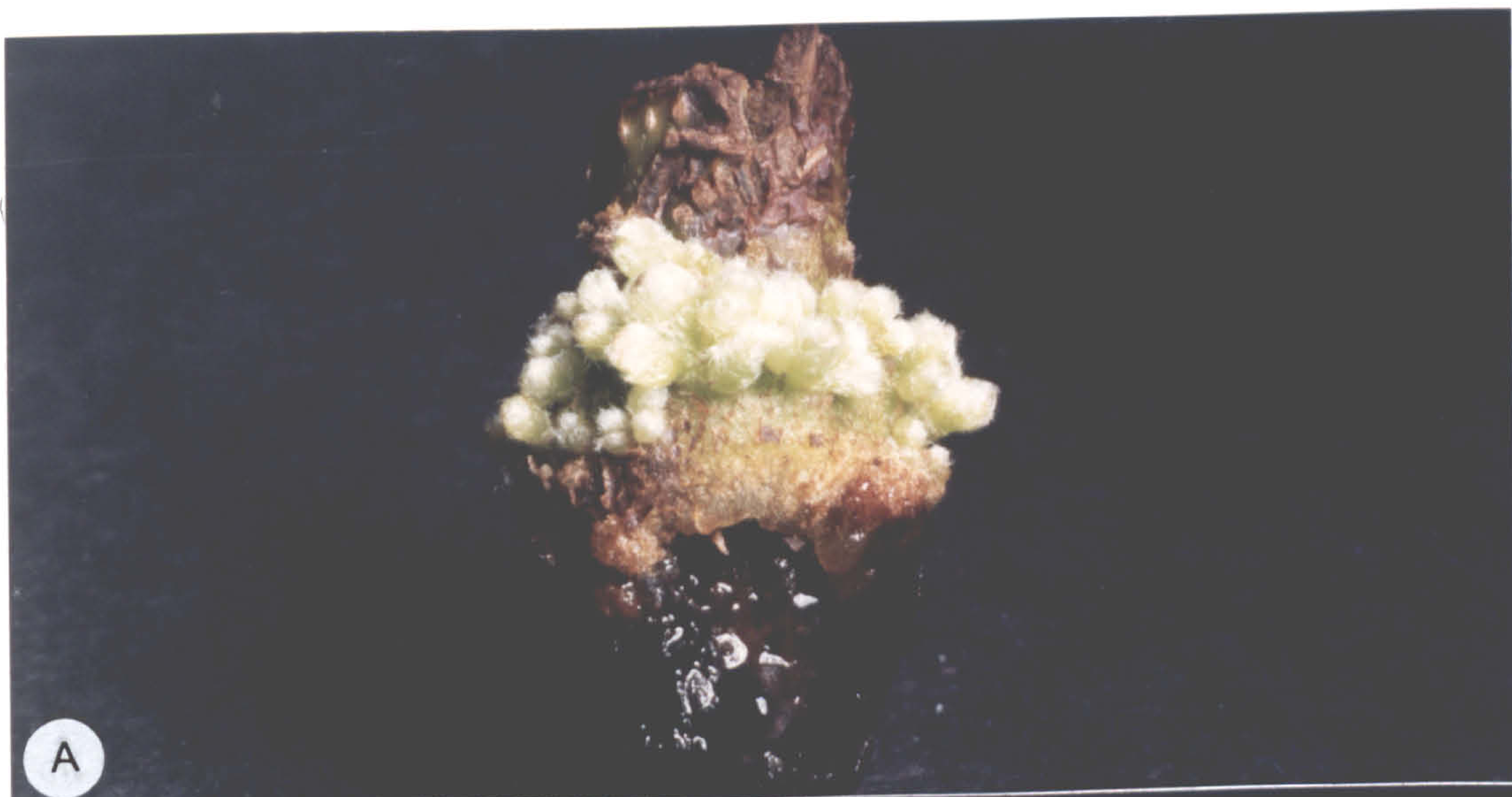
## PLATE : 7.01

*Annona squamosa* L. : multiple shoot induction from nodal segments. Medium used : MS + BAP (1.0 $mgl^{-1}$) + NAA (0.1 $mgl^{-1}$). Culture vessel volume = 60 $cm^3$. Each vessel contained one cutting. Cultures grown at *ca.* 25°C in continuous light; PAR = 80 $\mu mol\ m^{-2}s^{-1}$.

(a) 5 weeks old culture: vessels were capped with polypropylene disc (X5); note the development of numerous buds.

(b) 7 weeks old culture; vessels were capped with polypropylene disc; note some buds were elongating and partially opening and some had already died (X5).

(c) 7 weeks old culture; vessels were capped with fast forced ventilation apparatus (flow rate = 5 $cm^3\ min^{-1}$); note elongated new shoots with expanding leaves *cf.* (b) (X3).


A

B

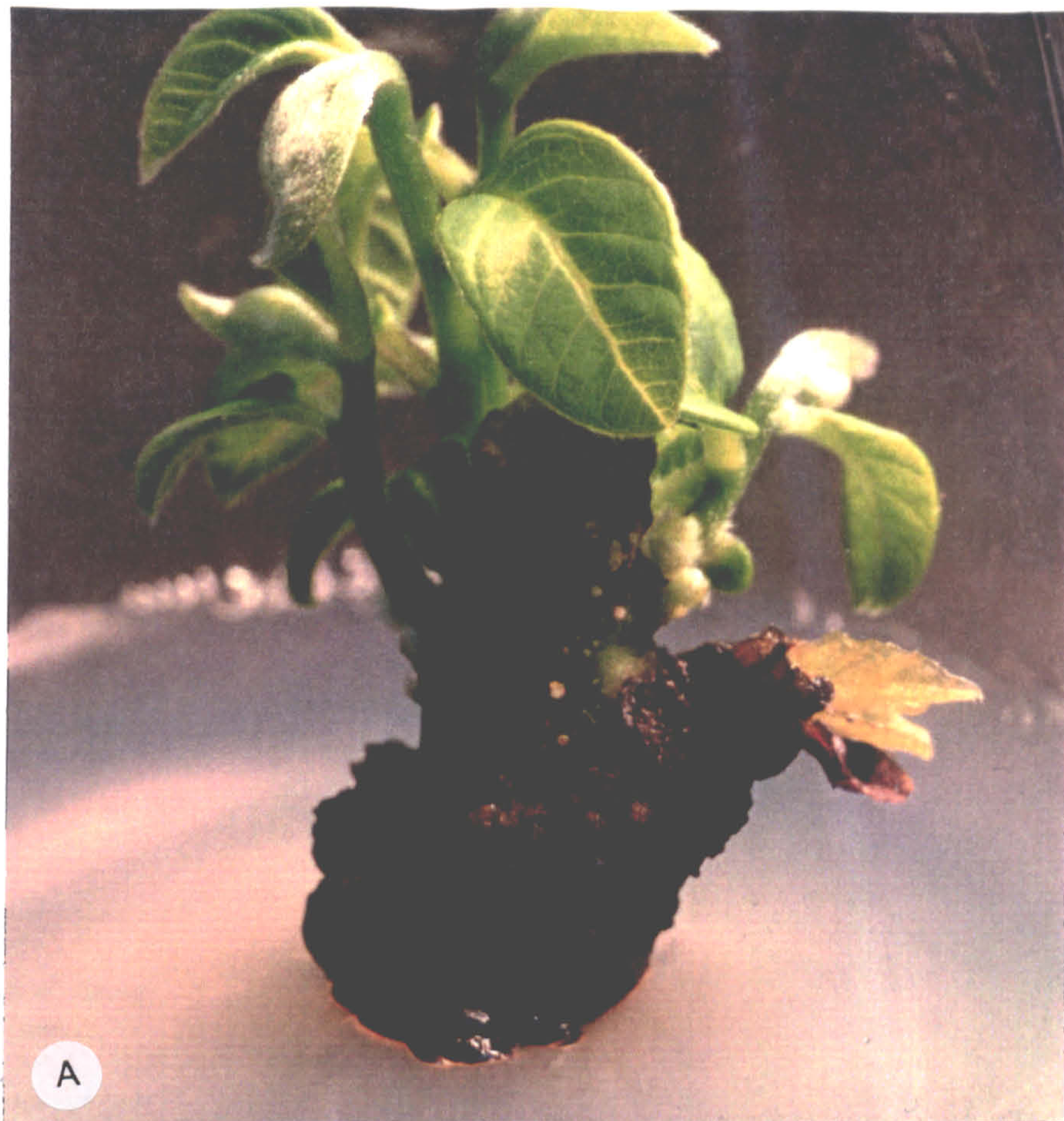
C

## PLATE : 7.02

*Annona squamosa* L. : multiple shoot induction from nodal segment. Medium used : MS + BAP (1.0 $mgl^{-1}$) + NAA (0.1$mgl^{-1}$). Culture vessel volume = 60 $cm^3$. Each vessel contained one cutting. Cultures grown at *ca.* 25°C in continuous light; PAR = 80 $\mu mol\ m^{-2}s^{-1}$.

(A) 10 weeks old culture; vessels were capped with fast forced ventilation apparatus (flow rate = 5 $cm^3\ min^{-1}$); note elongated shoots with large leaf area (X5).

(B) 7 weeks old culture; explants cultured under diffusive ventilation (polypropylene disc) for 25 days and then transferred to fast forced ventilation; note numerous shoots which were shorter and with smaller leaf areas than in (A) (X5).


A

B

sealed containers, 5.8 - 5.7 μl $l^{-1}$ in sealed containers with exogenous ethylene, 0.03 -0.05 μl $l^{-1}$ in the diffusive condition and no detectable amounts with forced ventilation.

**Table 7.04.** **Effects of different types of capping on multiple shoot induction of *Annona muricata* after 7 weeks of culture.**

| Characteristics | Sealed with silicone rubber bung | ❖Sealed + injected ethylene | Diffusive ventilation (polypropylene disc) | Forced ventilation (flow rate = 5.0 $cm^3$) | Grown under diffusive vent. and than transferred to forced ventilation |
|---|---|---|---|---|---|
| Days to bud initiation | --- | No buds developed | 7 -12 | 15 | --- |
| Number of buds per plant | --- | --- | 5.6 ± 0.8 | 4.1 ± 1.0 | 5.2 ± 0.9 |
| % of buds elongated | --- | --- | 43.5 | 83.1 | 73.9 |
| †Length of young shoots (mm) | --- | --- | 9.3 ± 0.4 | 17.5 ± 2.1 | 14.3 ± 2.1 |
| No. of leaves per young shoot | --- | --- | 3.5 ± 0.3 | 5.1 ± 0.4 | 4.7 ± 0.8 |
| Leaf area per young shoot ($cm^2$) | --- | --- | 1.1 ± 0.2 | 2.6 ± 0.3 | 2.0 ± 0.4 |
| No. of nodes per young shoot | --- | --- | 1.1 ± 0.1 | 3.1 ± 0.5 | 1.9 ± 0.3 |

†mean maximum length of 5 shoots from each tube.
❖10 $cm^3$ of 23 μl $l^{-1}$ ethylene in air was injected.
*60 $cm^3$ vessels. each containing one cutting. Cultures grown at *ca.* 25°C in continuous light; PAR = 80 μmol $m^{-2}s^{-1}$. Each value represents a mean ± SE of 5 replicates. Elongated buds become young shoots.

On balance the growth from explants was appreciably better under forced ventilation than under diffusive ventilation only. The results also demonstrate very clearly that the sealed system is very detrimental for both species of *Annona*. There are strong indications that they are adversely affected by ethylene and that especially forced ventilation improves growth and development because of its flushing effect on the culture vessels.

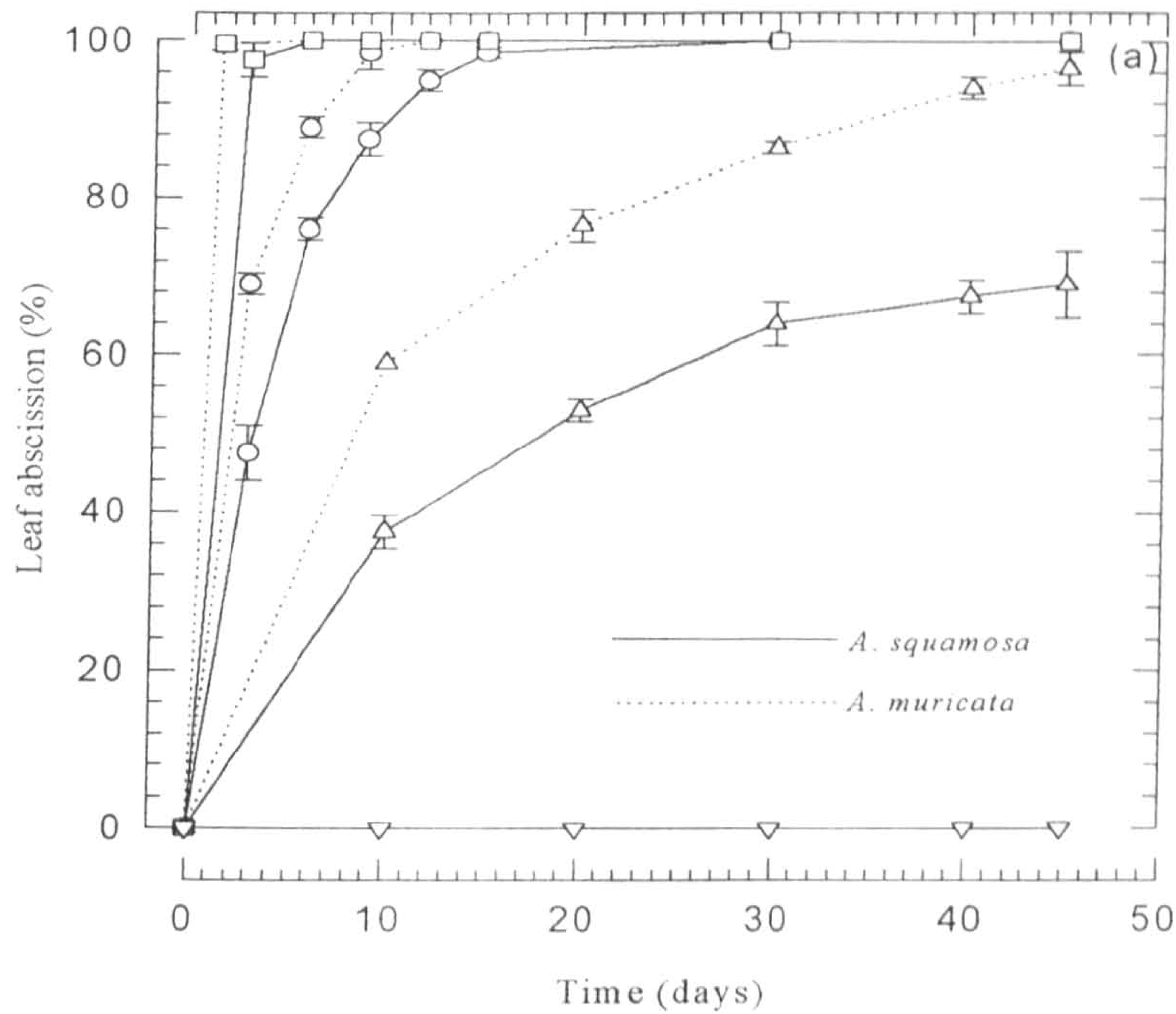
### 7.3.3. Effects of different ventilation types on leaf abscission

After 45 days of culture, forced ventilation proved to have been by far the most effective treatment to prevent leaf abscission: both *A.squamosa* and *A.muricata* showed no leaf abscission at all with FF-ventilation.

For plantlets cultured under diffusive ventilation the percentage of leaf abscission was 69% for *A. squamosa* and 95% for *A. muricata* after 45 days of culture (Fig 7.02a). In sealed conditions almost 100% abscission was recorded within only 10 and 15 days of culture in *A. muricata* and *A. squamosa* respectively. As expected, where plantlets were cultured in sealed vessels with ethylene added to the head-space, all the leaves of the cultured explants of both species dropped within 3-5 days.

The ethylene concentration above the culture was also measured during the experiment, and in the sealed vessels it was 1.01 $\mu l\ l^{-1}$ and 0 93 $\mu l\ l^{-1}$ in *A.squamosa* and *A.muricata* respectively at the end of the experiment. Culture vessels containing plantlets grown under diffusive ventilation accumulated very low concentrations of ethylene (0.06 $\mu l\ l^{-1}$ and 0.04 $\mu l\ l^{-1}$ in *A.squamosa* and *A.muricata* respectively but it seemed to have been sufficient to induce physiological changes like leaf abscission. As expected with the forced ventilation no ethylene was accumulated in the vessels during the experimental period (Fig. 7.02b).

These results are consistent with the leaf abscission phenomena found in this experiment, i.e. the higher the concentration of the accumulated ethylene (exogenous or endogenous) in the culture atmosphere the greater was the abscission rate of the species. Similar findings were reported by Lemos and Blake (1994) where leaf abscission *A.squamosa* was reduced by using an ethylene inhibitor. They also recorded that in vessels capped with cling film and polypropylene discs this species showed 86% and 70% leaf abscission after 4 weeks of culture. Ethylene-induced leaf abscission in *A. squamosa* was also very recently reported by (Amstrong *et al.* 1996), where forced ventilation and the ethylene inhibitor (STS) were introduced to control abscission. Earlier, in 1973, Abeles also showed that ethylene is particularly important in the



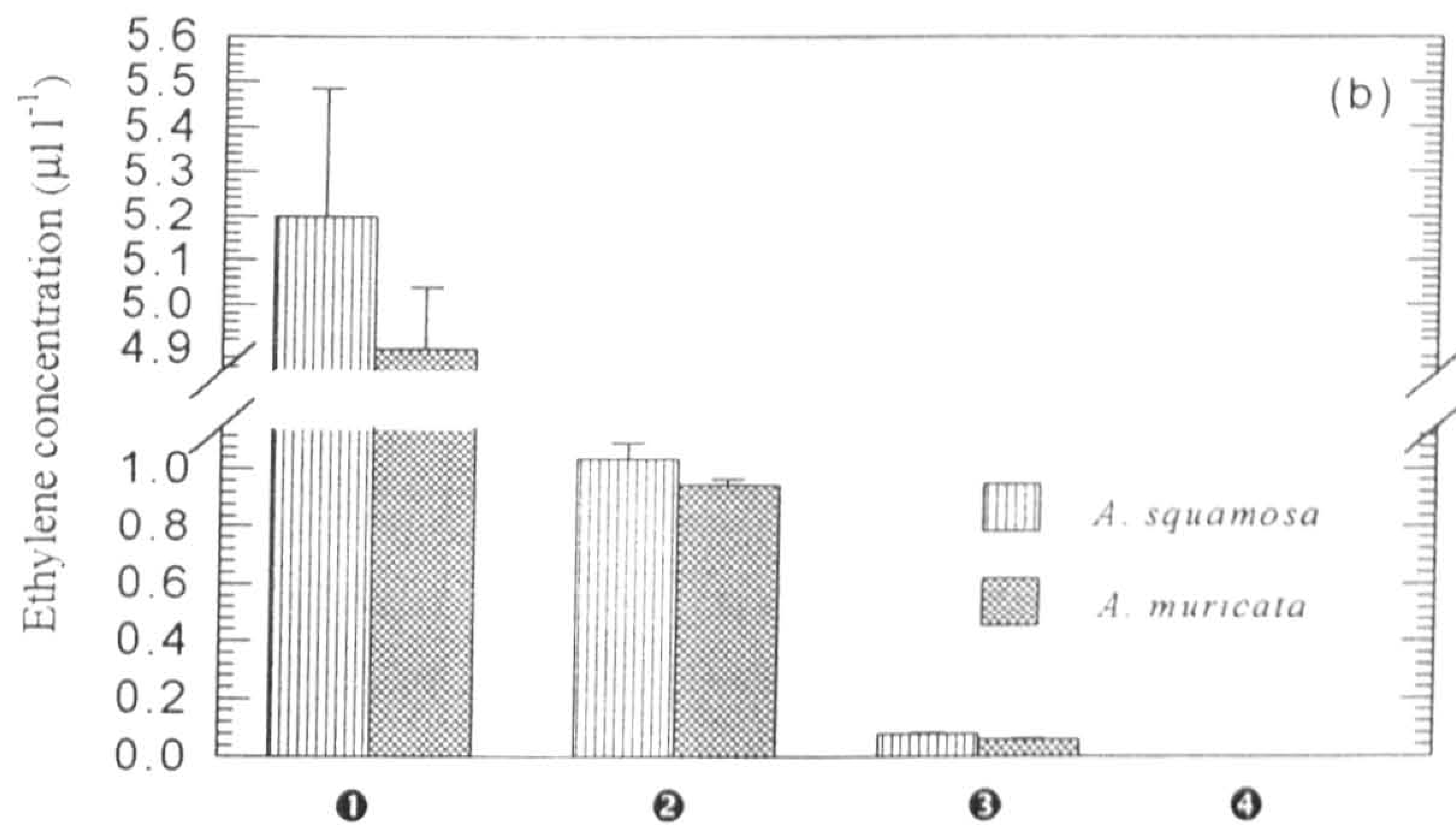




**Fig. 7.02.** Effects of different types of 'capping' on (a) percentage of leaf abscission and (b) ethylene concentrations (after 45 days of treatment) in the head-space of *Annona squamosa* L. and *A. muricata* L. cultures in 60 $cm^3$ vessels; cultures were grown under continuous light at 80 µmol $m^{-2}$ $s^{-1}$ (PAR), 25°C and 21% RH. Each vessel contained only one plantlet. Each symbol represents a mean ± SE of 5 - 10 replicates. ❶ & ○ = sealed with silicone rubber bung; ❷ & □ = sealed with silicone rubber bung and 10 $cm^3$ ethylene (23 µl $l^{-1}$ ethylene in air) was injected in each vessels immediately after sealing; ❸ & Δ = capped with polypropylene disc (diffusive ventilation) and ❹ & ∇ = capped with fast forced (FF) ventilation apparatus (flow rate = 5 $cm^3$ $min^{-1}$). Plantlets were previously grown under FF-ventilation apparatus for 14 days.

sequence of abscission phenomena. Therefore, it seems reasonable to conclude that accumulated ethylene is responsible for the leaf abscission of *in vitro* grown *Annona squamosa* and *A. muricata* plantlets and that it can be successfully controlled by FF-forced ventilation.

### 7.3.4. Flower-bud development in *Annona muricata*

#### 7.3.4.1. Flower-bud initiation

Firstly, a variety of hormonal treatments (12) were devised to try and initiate flower-bud development in *Annona muricata*; of these only five were successful (Table 7.05).

The best performance was observed using medium 1 [MS+BAP (2.0 mg $l^{-1}$)+CH (2.0 g $l^{-1}$)] in which, within 35 days of culture, flower-buds were initiated from the tips of the shoots (Plate 7.03a). Eventually a mean of 4.7 flower-buds were produced with a fresh weight of *ca.*48 mg per flower-bud (Plate 7.03b). When CH was not added in the medium (media 3, 4 and 5) fresh weights of the flower-buds were very significantly less (Table 7.05). There was also some indication that an increase or decrease in BAP concentration (media 4 and 5) reduced flower bud numbers.

**Table 7.05. Effects of different hormonal treatments on *in vitro* flower-bud development in *Annona muricata* after 60 days of culture.**

| *Media used | % of tubes with flower-bud | Dates of flower-bud initiation | No. of flower-buds | Fresh weight of each flower-bud (mg) |
|---|---|---|---|---|
| Medium - 1 | 81.0 | 35-40 | 4.7±0.2 | 47.6±4.5 |
| Medium - 2 | 78.9 | 40-45 | 4.5±0.2 | 40.1±2.9 |
| Medium - 3 | 67.1 | 40-45 | 4.3±0.3 | 30.1±5.1 |
| Medium - 4 | 66.9 | 40-45 | 3.3±0.5 | 30.3±3.5 |
| Medium - 5 | 71.8 | 50-60 | 2.8±0.1 | 29.5±4.0 |

***Medium - 1** : MS + BAP (2.0 mg$l^{-1}$) + CH (2.0 g$l^{-1}$); **Medium - 2** : MS + BAP (2.0 mg$l^{-1}$) + CH (1.0 g$l^{-1}$); **Medium - 3** : MS + BAP (2.0 mg$l^{-1}$); **Medium - 4** : MS + BAP (1.0 mg$l^{-1}$); **Medium - 5** : MS + BAP (2.5 mg$l^{-1}$).

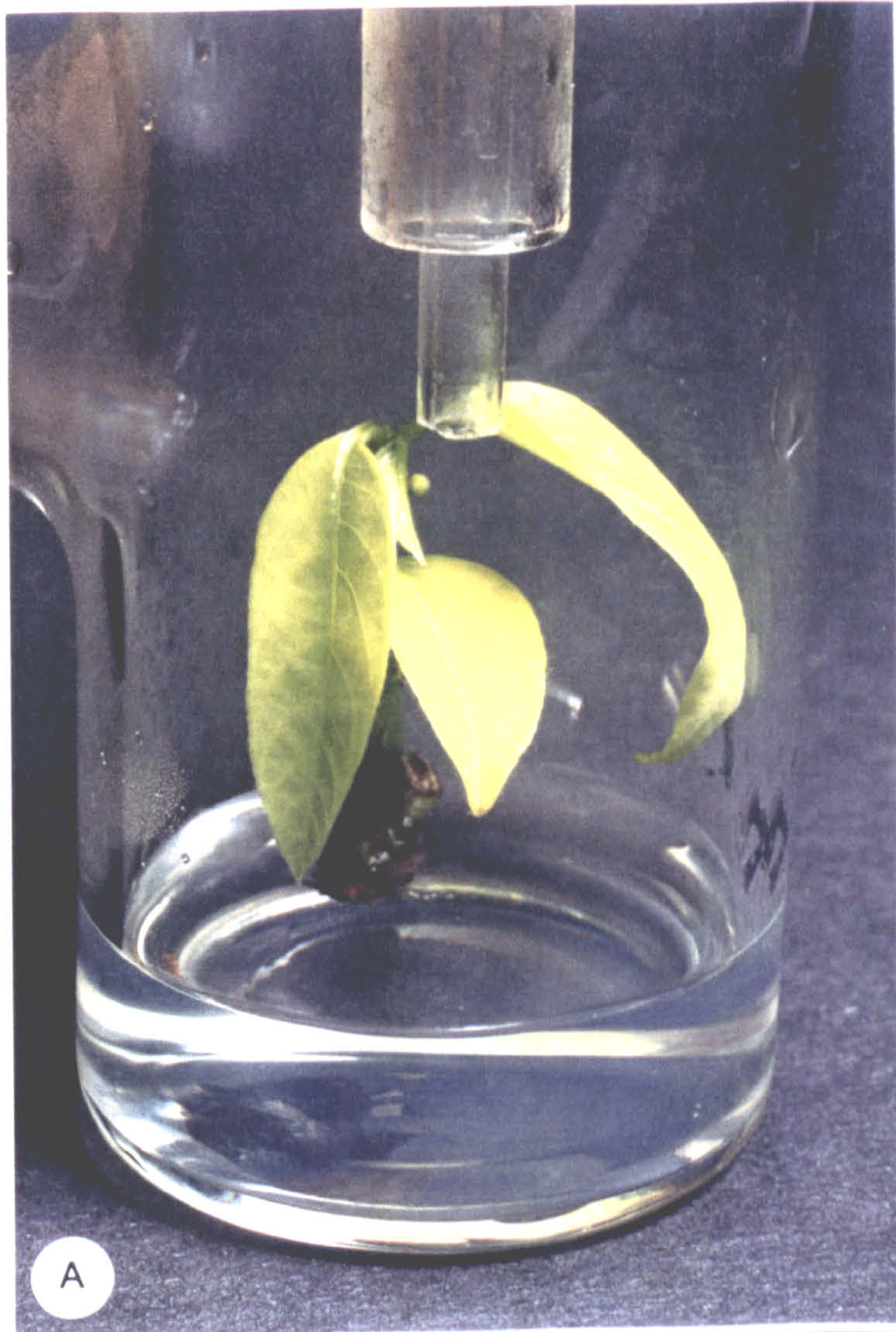
†60 $cm^3$ vessels, each containing one cutting, were capped with polypropylene disc. Cultures grown at *ca.* 25°C in continuous light; PAR = 80 $\mu mol\ m^{-2}s^{-1}$. Each value represents a mean ± SE of 5 replicates.

## PLATE : 7.03

*Annona muricata* L. : flower-bud development from nodal segments. Medium used : MS + BAP (2.0 $mgl^{-1}$) + CH (1.0 $gl^{-1}$). Culture vessel volume = 60 $cm^3$. Each vessel contained one cutting and capped with fast forced ventilation (flow rate = 5 $cm^3\ min^{-1}$) apparatus. Cultures grown at *ca.* 25°C in continuous light; PAR = 80 $\mu mol\ m^{-2}s^{-1}$.

(A) 5 weeks old culture; note flower-bud initiation from the tip of the shoot (X2.3).

(B) 2 weeks old flower-buds of *Annona muricata* L developed *in vitro* (X10).


A

B

#### 7.3.4.2. Effects of different types of ventilation on flower-bud abscission and development

Compared with all other treatments, the forced ventilation (5.0 $cm^3$ $min^{-1}$) showed the best performance in controlling abscission of *in vitro* grown flower-buds: *per cent* abscission was zero (Fig. 7.03; Plate 7.04a). After 21 days of culture a mean of 3.1 new flower-buds had developed and this was 2.6X higher that of diffusive ventilation. Also, the total fresh weight of flower-buds was significantly greater (1.7X) than in the diffusive treatment (Table 7.06).

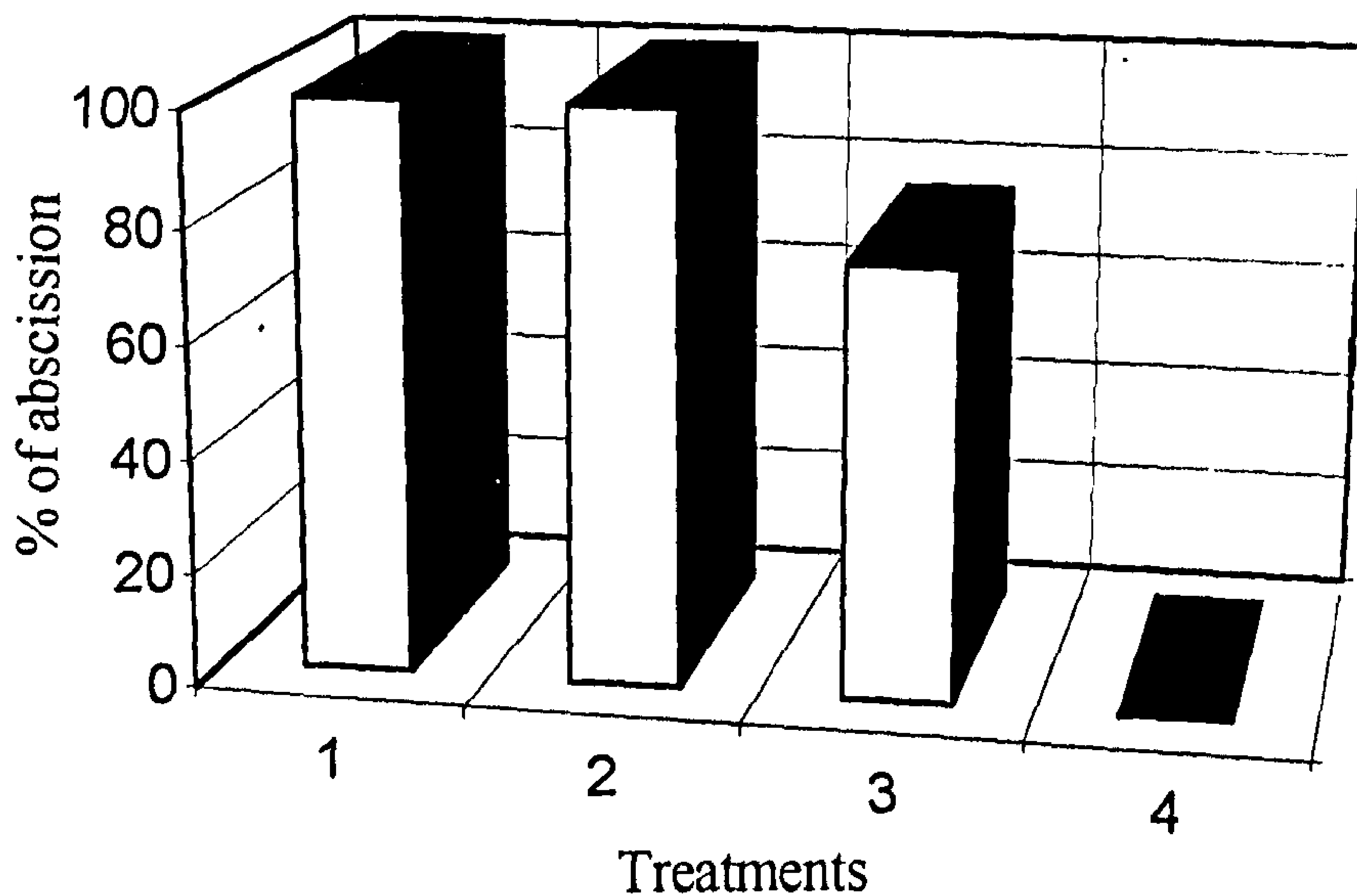



**Fig. 7.03.** Effects of different types of 'capping' on percentage of flower-bud abscission (after 21 day of treatment) of *Annona muricata* L. cultures in 60 $cm^3$ vessels; cultures were grown under continuous light at 80 µmol $m^{-2}$ $s^{-1}$ (PAR), 25°C and 21% RH. Each vessel contained only one plantlet. Each symbol represents a mean ± SE of 5 - 10 replicates. ❶ = sealed with silicone rubber bung; ❷ = sealed with silicone rubber bung + 10 $cm^3$ ethylene (23 µl $l^{-1}$ ethylene in air) was injected in each vessel immediately after sealing; ❸ = capped with polypropylene disc (diffusive ventilation) and ❹ = capped with fast forced (FF) ventilation apparatus (flow rate = 5 $cm^3$ $min^{-1}$). Plantlets had been previously grown under FF-ventilation apparatus for 20 days.

Under diffusive ventilation a total of 3.2 (mean) flower-buds had been observed of which three-quarters had dropped by the end of the experiment (Fig. 7.03; Plate 7.04b). In the sealed vessels 100% flower-bud abscission was observed and plants ultimately died by the end of the experiment. Similarly, in presence of exogenous ethylene in the

## PLATE : 7.04

*Annona muricata* L.: flower-bud development from nodal segments. Medium used : MS + BAP (2.0 $mgl^{-1}$) + CH (1.0 $gl^{-1}$); Culture vessel volume = 60 $cm^3$. Each vessel contained one cutting. Cultures grown at *ca.* 25°C in continuous light; PAR = 80 $\mu mol\ m^{-2}s^{-1}$.

(A) four weeks old culture grown under fast forced (FF) ventilation (flow rate = 5 $cm^3\ min^{-1}$); note flower-buds were not dropped in this treatment (X2.4).

(B) four weeks old cultures (3 weeks under FF ventilation and then transferred to diffusive ventilation (capped with polypropylene disc); note some flower- buds and leaves has already dropped (X3).

A

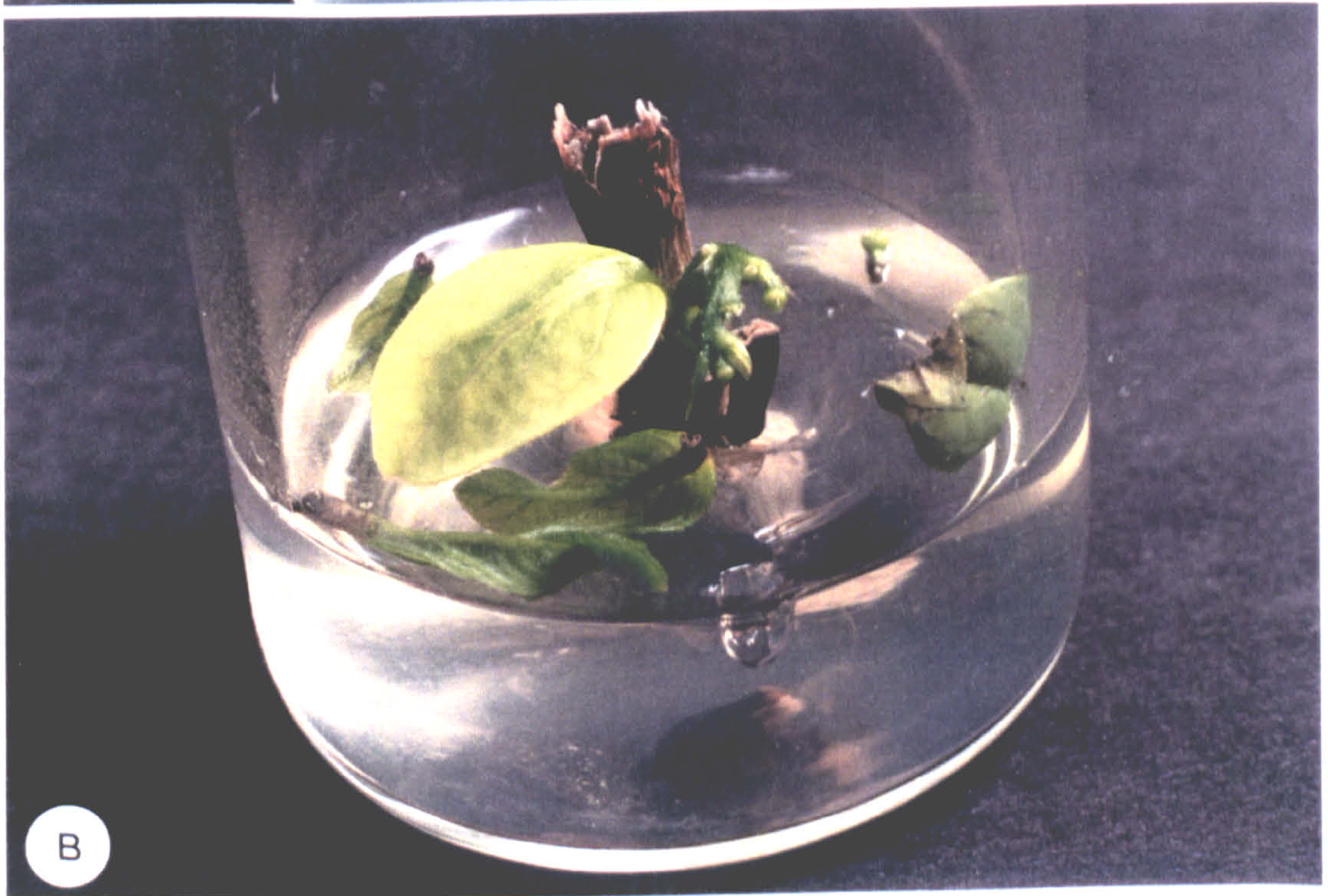

B

**Table 7.06.** **Effects of different types of ventilation on flower-bud development and fresh weight of *Annona muricata*.**

| Treatments | Number of flower-bud | | | Total fresh weight of flower-buds (mg) | |
|---|---|---|---|---|---|
| | ✤Initial number | New flower-buds developed | †Final number | ✤Initial weight | †Final weight |
| **Sealed vessels** | 2.2±0.3 | 0.0 | --- | 32.3±3.3 | --- |
| **❖Sealed + injected ethylene** | 2.1±0.2 | 0.0 | --- | 33.0±5.1 | --- |
| **Diffusive ventilation** | 2.0±0.2 | 1.2±0.3 | 3.2±0.2 | 31.8±2.7 | 66.7±6.4 |
| **Forced ventilation** | 2.1±0.4 | 3.1±0.2 | 5.2±0.6 | 33.7±4.3 | 113.5±9.3 |

†After 21 days of treatment (including dropped flower-buds); plantlets had been previously grown with fast flow ventilation for 20 days.

✤Initial numbers and weights of flower-buds were taken from extra plans harvested at the beginning of experiment.

❖10 $cm^3$ of 23 $\mu l\ l^{-1}$ ethylene in air was injected.

*60 $cm^3$ vessels, each containing one cutting. Cultures grown at *ca.* 25°C in continuous light; PAR = 80 $\mu mol\ m^{-2}s^{-1}$. Flow rate of forced ventilation (fast flow) was 5 $cm^3\ min^{-1}$; for sealed and diffusive ventilation, vessels were capped with silicone rubber bungs and polypropylene discs respectively. Each value represents a mean ± SE of 5 replicates.

atmosphere of the sealed culture vessels all the flower-buds (100%) abscised, but buds continued to be formed throughout the experiment. As in the sealed controls, plantlets cultured in this treatment became brown in colour with some necrosis and ultimately died. It should be mentioned that in the latter two treatments almost all the buds had dropped within only three days of the start of the experiment (data not shown).

The ethylene concentrations in the head-space of the culture vessels were also measured at the end of the experiment. As shown in Fig. 7.04 the ethylene concentration in sealed conditions was 0.59 $\mu l\ l^{-1}$; under diffusive ventilation although it was quite low (0.035 $\mu l\ l^{-1}$) it was still apparently sufficient to alter the physiological activity in the plant. A complete absence of ethylene was noted in forced ventilated vessels.

It has been reported elsewhere that low levels of ethylene promote abscission of fruits, leaves and buds of many species (Sisler and Yang 1984). Although flower-bud abscission of *in vitro* grown plantlets have not previously been described, many authors have shown that the treatment of young flower-buds of plants such as carnation and morning glory with external ethylene accelerated senescence (Yang and Hoffman 1984) and ultimately terminated the functional life of the flower-buds. However, these findings does not tell us anything about the abscission of *in vitro* grown flower-buds. In the case of *A.squamosa*, the ethylene data above accord with pattern found in the degree of *in vitro* grown flower-bud abscission, and taken in conjunction with the literature reported above they suggest a causal link with accumulated ethylene.

### 7.3.5. Effects of different types of ventilation on growth, photosynthetic rate, and chlorophyll and carotenoid contents

#### 7.3.5.1. Growth

***Annona squamosa*:** From a consideration of all the parameters of growth and development of plantlets in the various treatments, it is evident that explants grown under forced ventilation were more vigorous than in the other treatments (Table 7.07). Thus, after 45 days of culture, the fresh weights and numbers of attached leaves were 139 mg and 13.5 respectively, which were 3.5X and 3X greater than those in diffusive

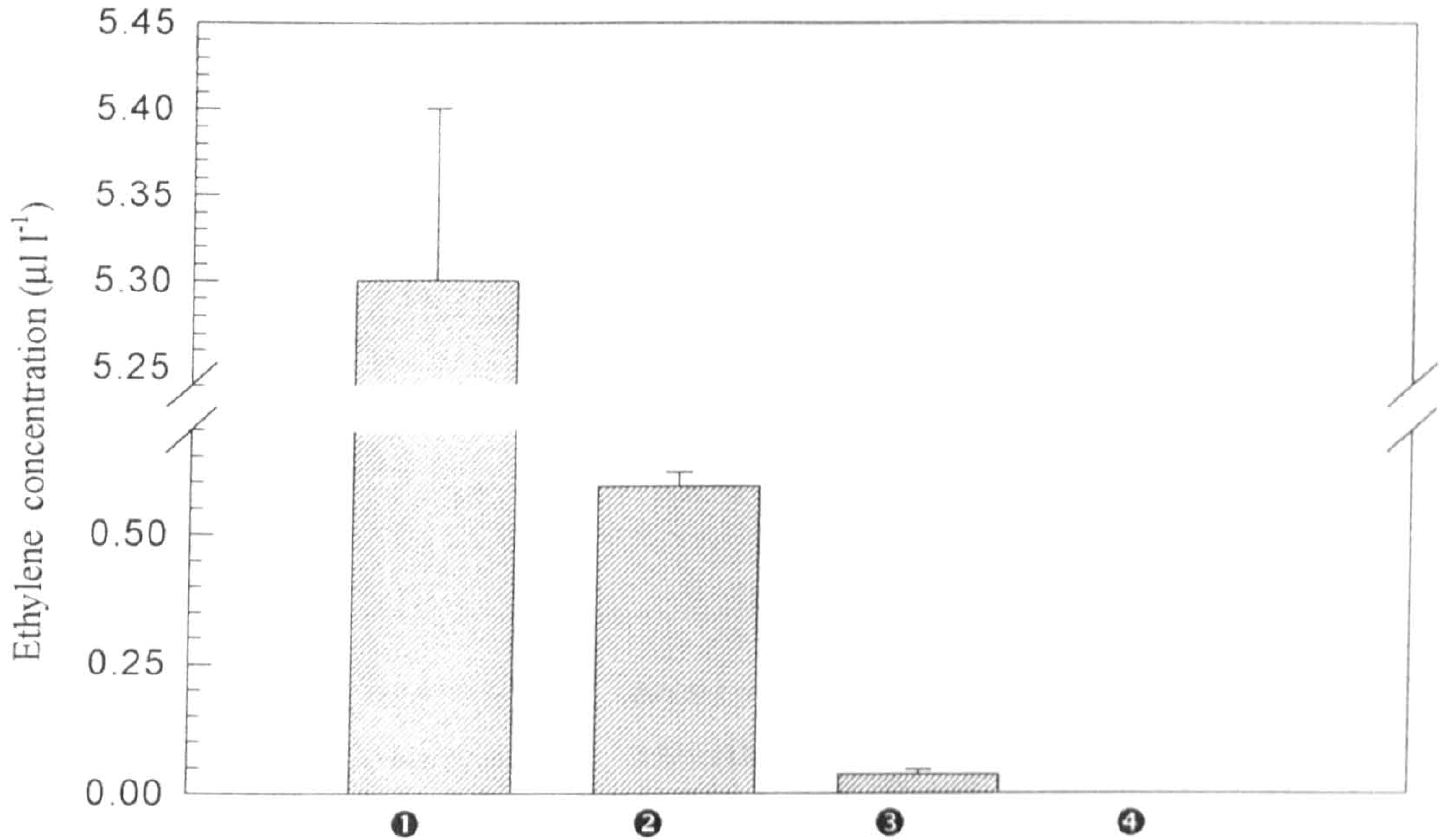



**Fig.7.04.** Effects of different types of 'capping' on ethylene concentrations in the head-space of *Annona muricata* L. cultures (after 21 day of treatment) in 60 $cm^3$ vessels, grown under continuous light at 80 µmol $m^{-2}$ $s^{-1}$ (PAR), 25°C and 21% RH. Each symbol represents a mean ± SE of 5 replicates. ➊ = sealed with silicone rubber bung; ➋ = sealed with silicone rubber bung and 10 $cm^3$ ethylene (23 µl $l^{-1}$ ethylene in air) was injected in each vessel immediately after sealing; ➌ = capped with polypropylene disc (diffusive ventilation) and ➍ = capped with fast forced ventilation apparatus (flow rate = 5 $cm^3$ $min^{-1}$). Plantlets had been previously grown under FF-ventilation apparatus for 20 days.

**Table 7.07.** Effects of different types of ventilation on growth and development of 45 days old *in vitro*-grown *Annona squamosa* cuttings.

| Characteristics | Forced ventilation | Diffusive ventilation | Sealed | ❖ Sealed + injected ethylene |
|---|---|---|---|---|
| **Leaves** | | | | |
| †Number | 13.5±2.3 | 12.8±1.1 | 1.02±0.5 | -- |
| Number on plants | 13.5±2.3 | 4.7±0.4 | 0.5±0.5 | -- |
| †Total FW (mg) | 139.3±14.3 | 123.5±8.9 | 10.5±1.9 | -- |
| FW (mg) on plant | 139.3±14.3 | 39.5±4.6 | 1.4±0.3 | -- |
| †Area ($cm^2$) | 12.5±1.8 | 9.3±1.2 | 0.9±0.2 | -- |
| **Stem** | | | | |
| Total FW (mg) | 287.8±15.9 | 259.5±12.8 | 109±1.8 | -- |
| Increase in FW (mg) | 188.3±10.2 | 160.2±9.9 | 10.1±0.9 | |
| Length (mm) | 13.3±1.1 | 9.9±1.0 | -- | -- |
| No. of new nodes | 5.3±0.7 | 3.9±0.3 | -- | -- |
| **Buds** | | | | |
| Total no. of buds | 5.1±1.3 | 6.0±0.9 | -- | -- |
| No. of buds opened | 4.7±0.6 | 3.1±0.7 | -- | -- |
| % of opened buds | 92.2 | 51.7 | -- | -- |

†Including the dropped leaves.
❖10 $cm^3$ of 23 $\mu l\ l^{-1}$ ethylene in air was injected.
*60 $cm^3$ vessels, each containing one cutting. Cultures grown at *ca.* 25°C in continuous light; PAR = 80 $\mu mol\ m^{-2}s^{-1}$. Flow rate of forced ventilation (fast flow) was 5 $cm^3\ min^{-1}$; for sealed and diffusive ventilation vessels were capped with silicone rubber bungs and polypropylene discs respectively. Each value represents a mean ± SE of 5 - 7 replicates.

ventilation; stem fresh weight was marginally higher as were the numbers of new nodes. The numbers of new buds (incuding those developed subsequently on the original explant and on the new shoot) were similar in the FF- and diffusive-ventilation treatments, but the numbers of these which opened was marginally higher in the FF-treatment and at least half of those in the diffusive treatment either remained unopened or died.

In sealed conditions there was little new growth of leaves or stems (Table 7.07), and by the end of the experiment most of the small leaves formed, or originally present, had fallen. Explants in sealed vessels with added ethylene developed no further shoot or leaf system and were dead after only 10 days.

***Annona muricata*** : The patterns of growth in this species under the different types of ventilating system were very similar to those of *A.squamosa*, except that there was no growth whatsoever in the sealed systems and the plants died in both (Table 7.08).

Healthy leaf and shoot systems were observed in the plantlets grown under forced ventilation: all the leaves survived and their numbers and fresh weights at the end of the experiment were 10.2 and 108.6 mg respectively, compared to 2 and 14.1 mg in the diffusive treatment where all but a fifth of the leaves had fallen. Again, stem fresh weight was more than with the diffusive treatment and the numbers of buds which opened was greater but perhaps not significant.

### 7.3.5.2. Photosynthesis

The relationships between net photosynthetic rate (μmol $m^{-2}$ leaf $s^{-1}$) and PAR (μmol $m^{-2}$ $s^{-1}$) are shown in Fig. 7.05. Here, it is evident that net photosynthetic rate per unit leaf area is highest in plantlets grown under forced ventilation, lowest in the sealed treatments (data for *A. squamosa* only), and intermediate under diffusive ventilation. Net photosynthetic rate in *A. squamosa* was 3.12 μmol $m^{-2}$ leaf $s^{-1}$ at PAR = 220 μmol $m^{-2}$ $s^{-1}$, which was 1.3X and 4.9X greater that in diffusive ventilation and sealed vessels respectively. However, at the normal growth room PAR (80 μmol $m^{-2}$ $s^{-1}$) it can be seen

**Table 7.08.** Effects of different types of ventilation on growth and development of 45 days old *in vitro*-grown *Annona muricata* cuttings.

| Characteristics | Forced ventilation | Diffusive ventilation | Sealed | Sealed + injected ethylene |
|---|---|---|---|---|
| **Leaves** | | | | |
| †Number | 10.2±2.0 | 9.7±1.3 | -- | -- |
| Number on plants | 10.2±2.0 | 2.0±0.3 | -- | -- |
| †Total FW (mg) | 108.6±18.4 | 91.3±6.8 | -- | -- |
| FW (mg) on plant | 108.6±18.4 | 14.1±0.9 | -- | -- |
| †Area ($cm^2$) | 9.5±1.6 | 6.3±1.0 | -- | -- |
| **Stem** | | | | |
| Total FW (mg) | 224.8±13.4 | 198.3±14.6 | - | -- |
| Increase in FW (mg) | 134.7±9.3 | 107.2±11.2 | | |
| Length (mm) | 10.9±1.4 | 7.1±0.6 | -- | -- |
| No. of new nodes | 3.8±0.5 | 3.5±0.4 | -- | -- |
| **Buds** | | | | |
| Total no. of buds | 5.0±1.0 | 5.6±0.5 | -- | -- |
| No. of buds opened | 3.6±0.6 | 2.3±0.7 | -- | -- |
| % of opened buds | 72.0 | 41.1 | -- | -- |

†Including the dropped leaves.

❖ 10 $cm^3$ of 23 $\mu l\ l^{-1}$ ethylene in air was injected.

*60 $cm^3$ vessels, each containing one cutting. Cultures grown at *ca.* 25°C in continuous light; PAR = 80 $\mu mol\ m^{-2}s^{-1}$. Flow rate of forced ventilation (fast flow) was 5 $cm^3\ min^{-1}$; for sealed and diffusive ventilation vessels were capped with silicone rubber bungs and polypropylene discs respectively. Each value represents a mean ± SE of 5 - 7 replicates.

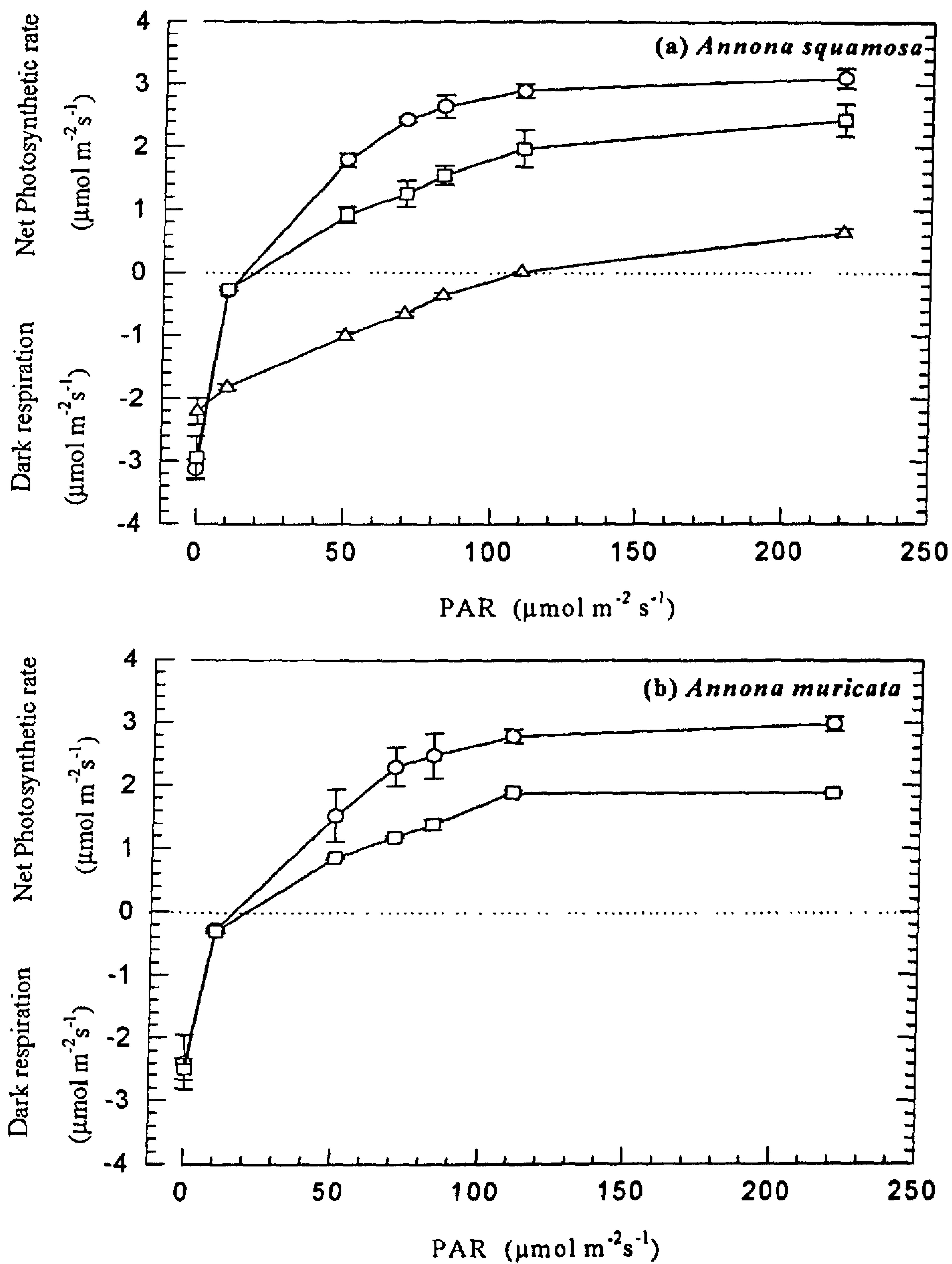



**Fig. 7.05.** Effects of different types of ventilation on net photosynthetic rates (based on leaf area) in different irradiances of (a) *Annona squamosa* L. and (b) *Annona muricata* L. after 45 days of culture in 60 $cm^3$ vessels; Δ = sealed with silicone rubber bung; □ = capped with polypropylene disc (diffusive ventilation) and O = capped with fast forced (FF) ventilation apparatus (flow rate = 5 $cm^3$ $min^{-1}$). Cultures had been previously grown under continuous light at 80 µmol $m^{-2}$ $s^{-1}$ (PAR), 25°C and 21% RH. Each vessel contained only one plantlet. Each symbol represents a mean ± SE of 4 - 5 replicates. Photosynthetic rates were measured at 350 µl $l^{-1}$ $CO_2$.

that net photosynthesis under FF-ventilation was 1.7X that with diffusive ventilation and that there was no net assimilation in the sealed condition; on the contrary, respiratory activity masked any photosynthetic assimilation at this light flux. This latter response is probably the result of there being few (and small) leaves but still a significant amount of original explant stem material.

Whether these photosynthetic rates reflect a difference in the photosynthetic fitness of the leaves from the various treatments is not unequivocally revealed by the current data. For example, since the stem fresh weights are similar in the FF and diffusive treatments, but the leaf numbers are much smaller with diffusive ventilation, the stem respiratory activity will exert a proportionately greater effect on the photosynthetic data from this treatment and lower the readings. It is interesting, however, that chlorophyll and carotenoid contents were generally higher under FF- than under diffusive-flow ventilation (see Section 7.3.7.3).

When photosynthesis rate is expressed on a *per* plantlet basis the greater vigour of the FF-treatment plants becomes even more evident (Fig. 7.06a ). Under normal growth room PAR (80 μmol $m^{-2}$ $s^{-1}$), the net photosynthetic rates with FF-ventilation were 15X those with diffusive ventilation. In the sealed condition no net assimilation took place.

In *A. muricata* the maximum photosynthetic rate in plantlets subjected to forced ventilation was 1.6X that in the diffusive treatment, but plantlets died only after 7 - 15 days of culture in the sealed system and as a consequence it was not possible to measure photosynthetic rates in this treatment

When photosynthesis rate is expressed on a *per* plantlet basis the greater vigour of the FF-treatment plants becomes even more evident (Fig. 7.06b). At 80 μmol $m^{-2}$ $s^{-1}$ PAR the net photosynthetic rate for plantlets grown under FF-ventilation was 24X that of those grown with diffusive ventilation Again it is interesting that chlorophyll and carotenoid contents in non-abscised leaves were generally higher under FF- than under diffusive-flow ventilation.

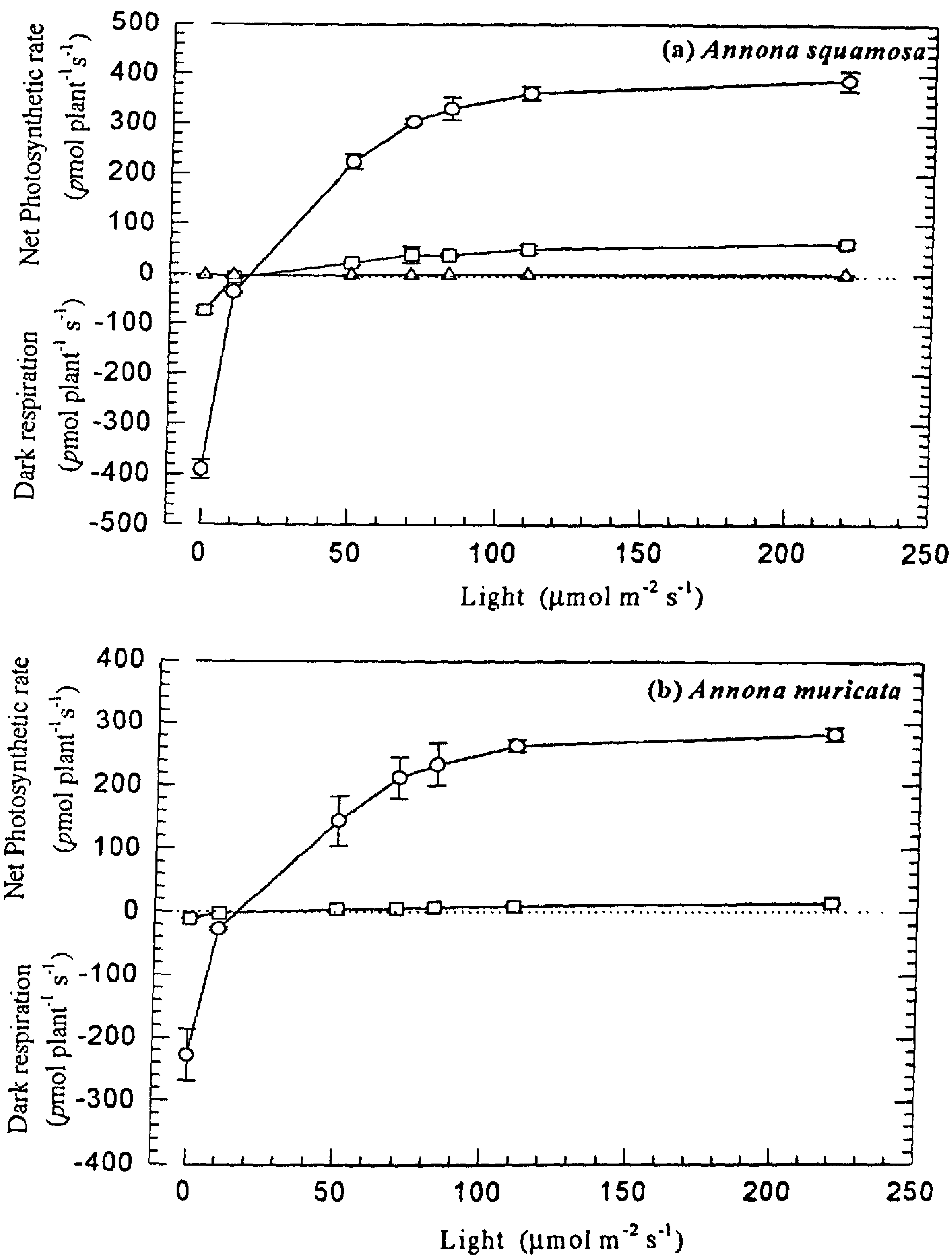



**Fig. 7.06.** Effects of different types of ventilation on net photosynthetic rate per plant in different irradiances of (a) *Annona squamosa* L. and (b) *Annona muricata* L. after 45 days of culture in 60 $cm^3$ vessels; Δ = sealed with silicone rubber bung; □ = capped with polypropylene disc (diffusive ventilation) and O = capped with fast forced (FF) ventilation apparatus (flow rate = 5 $cm^3$ $min^{-1}$). Cultures had been previously grown under continuous light at 80 µmol $m^{-2}$ $s^{-1}$ (PAR), 25°C and 21% RH. Each vessel contained only one plantlet. Each symbol represents a mean ± SE of 4 - 5 replicates. Photosynthetic rates were measured at 350 µl $l^{-1}$ $CO_2$.

Further observations on photosynthesis under the various ventilation systems are to be found in section 7.3.8.2.

#### 7.3.5.3. Chlorophyll and Carotenoid contents

In the non-abscised leaves, plantlets of *A squamosa* had higher chlorophyll contents under forced ventilation than under diffusive ventilation but the differences were not very significant. In *A. muricata,* on the other hand, the differences were greater and significant, the chlorophyll b being 1.6x greater in the FF-treatment (Fig. 7.07). The nearest comparison found in the literature concerns *Solanum tuberosum*: Cournec *et al.* (1991) showed that diffusive aeration of culture vessels increased chlorophyll contents above those in sealed vessels.

Carotenoid contents of FF- and diffusively-ventilated *A squamosa* were similar whilst those of *A. muricata* were very different: under forced ventilation they 1.3X greater It should be mentioned that due to the lack of leaves in the sealed vessels (with and without exogenous ethylene) it was not possible to determined the chlorophyll and carotenoid contents.

In the case of *A.muricata* it is possible that the lower chlorophyll and carotenoid levels in the diffusive treatment are a reflection of the effects of the endogenous ethylene found in this treatment.

### 7.3.6. Effects of different types of ventilation on the gas composition of culture vessel head-space

In these experiments the same plants were subjected to the three ventilation systems and hence none of the differences observed are attributable to differences in plant morphology.

#### 7.3.6.1. Ethylene

In sealed containers ethylene concentrations increased rapidly and reached peak levels (1.3 $\mu l\ l^{-1}$ in *A. squamosa.* and 1.15-1.17 $\mu l\ l^{-1}$ in *A. muricata*) within only 24 - 48 hours of culture For both the species the pattern of the changes in ethylene concentration

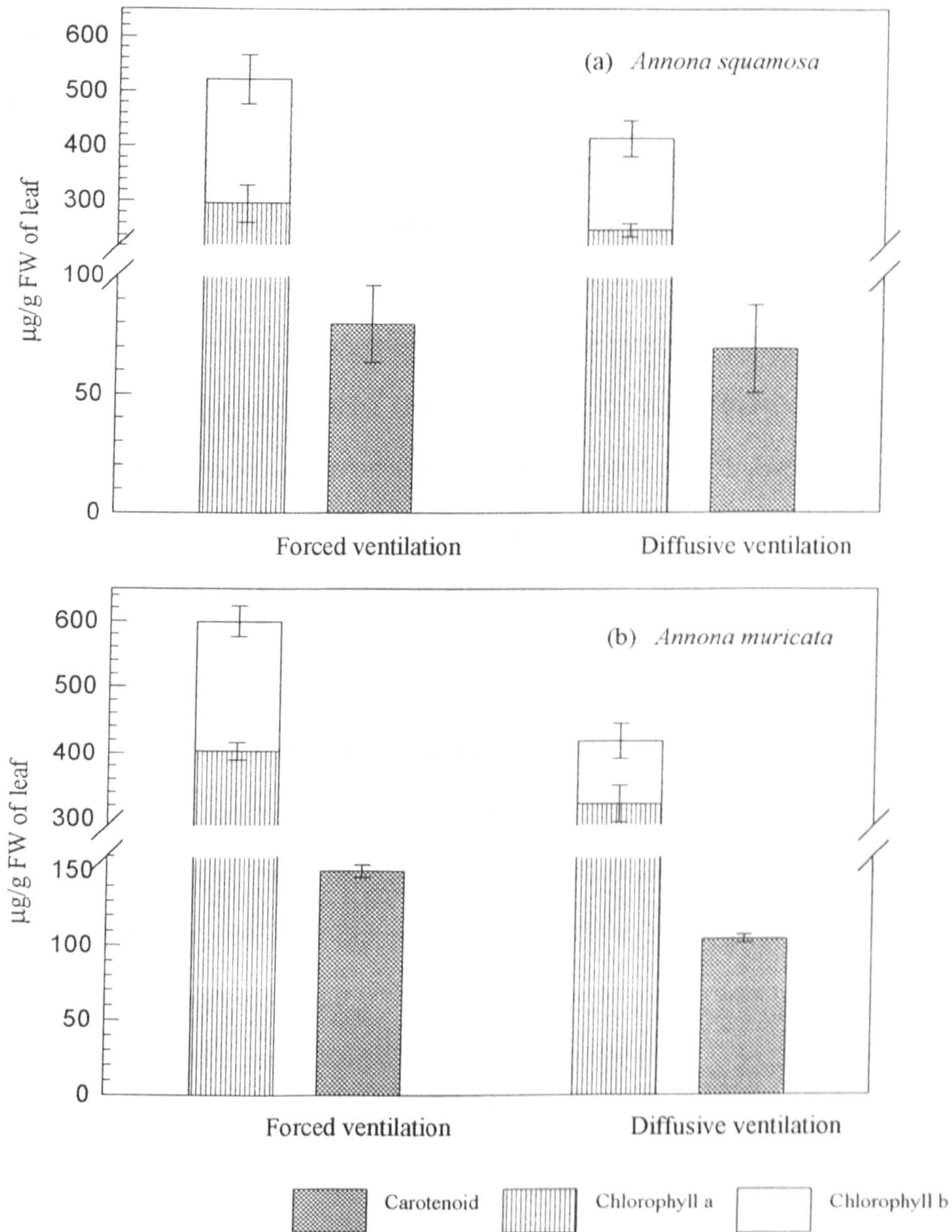



**Fig. 7.07.** Effects of diffusive (polypropylene disc) and forced ventilation (fast flow; flow rate = 5.0 $cm^3$ $min^{-1}$) on chlorophyll (a & b) and carotenoid contents of (a) *Annona squamosa* L. and (b) *Annona muricata* L. after 45 days of culture in 60 $cm^3$ vessels. Cultures grown under continuous light at 80 μmol $m^{-2}s^{-1}$ (PAR), 25°C and 21% RH. Each vessel contained only one plantlet. Each bar represents a mean ± SE of 4 - 5 replicates.

under diffusive ventilation was similar to that in the sealed vessels (Fig 7.08), but the peak concentration was only 0.122 μl $l^{-1}$ for *A. squamosa.* and 0.113 μl $l^{-1}$ for *A. muricata.* Under forced ventilation no ethylene accumulation was observed in the culture vessels, probably because any ethylene (or other toxic gases) produced by the plantlets was flushed out by the FF-ventilation.

It is interesting that even in the sealed vessels ethylene concentrations reached an equilibrium: in this case net production also ceased rather abruptly and this strongly suggests the switching-in of a negative feed-back mechanism. It is also interesting that the potential for ethylene production by a separate batch of plantlets did not diminish significantly with time over 3 days (Fig. 7.08b): repeated re-exposure to air followed by re-sealing resulted in similar production rates and peak levels. However, although the ethylene production did not noticeably diminish in this time the experiment was concluded because of leaf-fall at *ca.* 80-90 hours.

### 7.3.6.2. Carbon dioxide

Results were similar for both *A.squamosa* and *A.muricata* (Fig. 7.09).

The $CO_2$ concentration in the sealed vessels rose rapidly during any dark period and fell rapidly at the onset of any subsequent photoperiod. By the end of the dark period the $CO_2$ was very high (approx. 1.5%), while in the light it reached approx. 40 μl $l^c$ and, because gas-exchange with the outside atmosphere was totally restricted, this represents the $CO_2$ compensation point for these plantlets.

Under diffusive ventilation the $CO_2$ concentration in the atmosphere of the culture vessels decreased during light period to approx. 88 μl $l^{-1}$ which was 2.4X that of sealed vessels and above the $CO_2$ compensation point (see Fig. 7.10). It should noted that the rate of photosynthesis at 88 μl $l^{-1}$ is significantly less than at atmospheric levels of $CO_2$; at 70 μ mol $m^{-2}$ $s^{-1}$ PAR, with atmospheric $CO_2$ (350 μl $l^{-1}$), the net photosynthetic rate was 1.26 μmol $m^{-2}$ $s^{-1}$ (leaf area); with 88 μl $l^{-1}$ $CO_2$, the rate was only 0.003 μmol $m^{-2}$ $s^{-1}$.

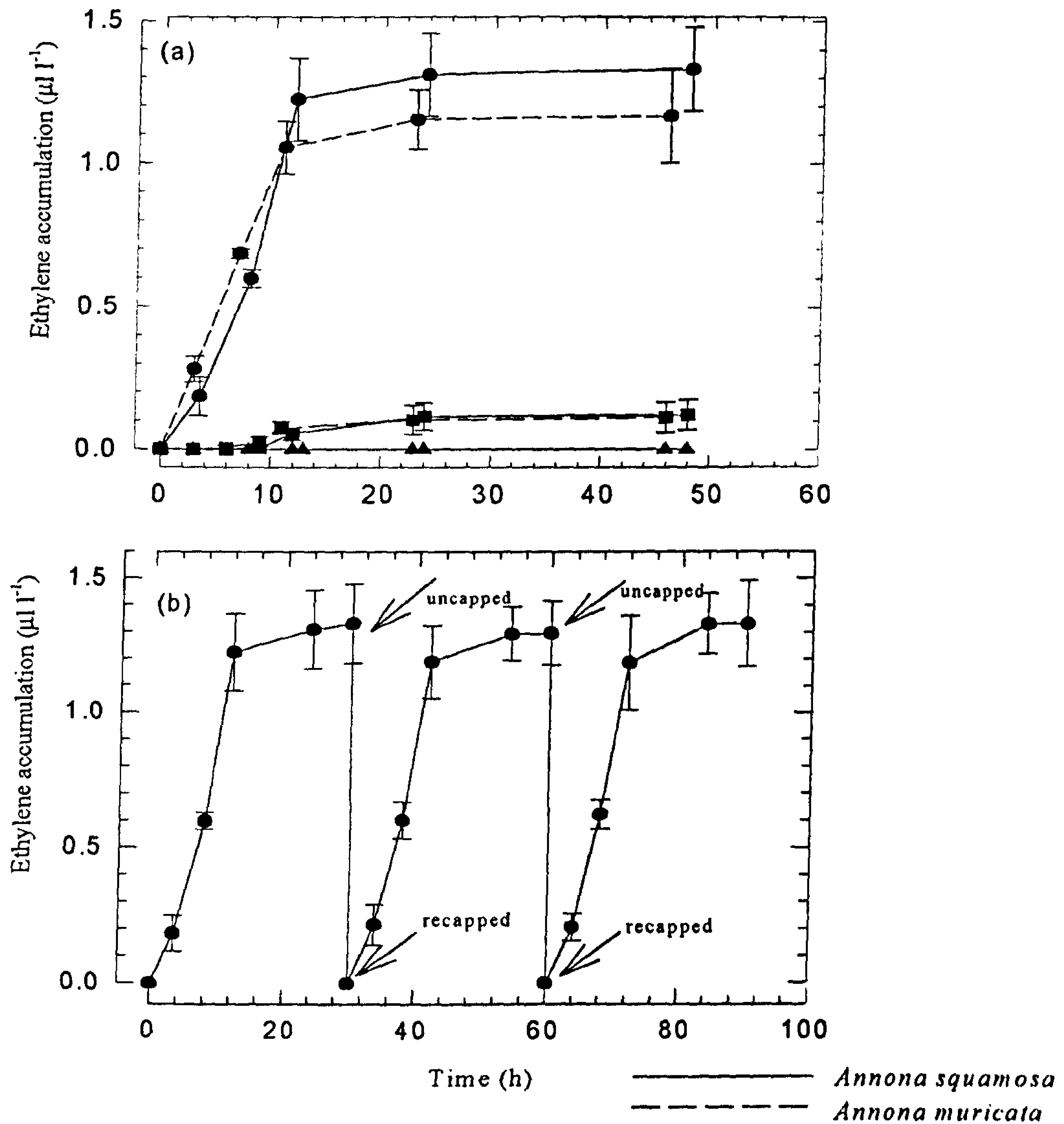



**Fig. 7.08.** (a) Effects of different types of ventilation on ethylene accumulation in the culture head-space of cuttings of *Annona squamosa* L. (leaf area = 8.5 $cm^2$; total fresh weight = 423 mg) and *Annona muricata* L. (leaf area = 7.9 $cm^2$; total fresh weight = 382 mg); (b) ethylene accumulation in the culture head-space of *Annona squamosa* L. (leaf area = 8.2 $cm^2$; total fresh weight = 416 mg) in sealed vessels. Vessels were uncapped, flushed with sterile air and recapped approx. every 30 hours.

45 days old cultures, previously grown in FF-ventilation in 60 $cm^3$ vessels; cultures were grown under continuous light at 80 µmol $m^{-2}$ $s^{-1}$ (PAR), 25°C and 21% RH. Each vessel contained only one plantlet. Each symbol represents a mean ± SE of 5 replicates. ● = sealed with silicone rubber bung; ■ = capped with polypropylene disc (diffusive ventilation) and ▲ = capped with fast forced (FF) ventilation apparatus (flow rate = 5 $cm^3$ $min^{-1}$).

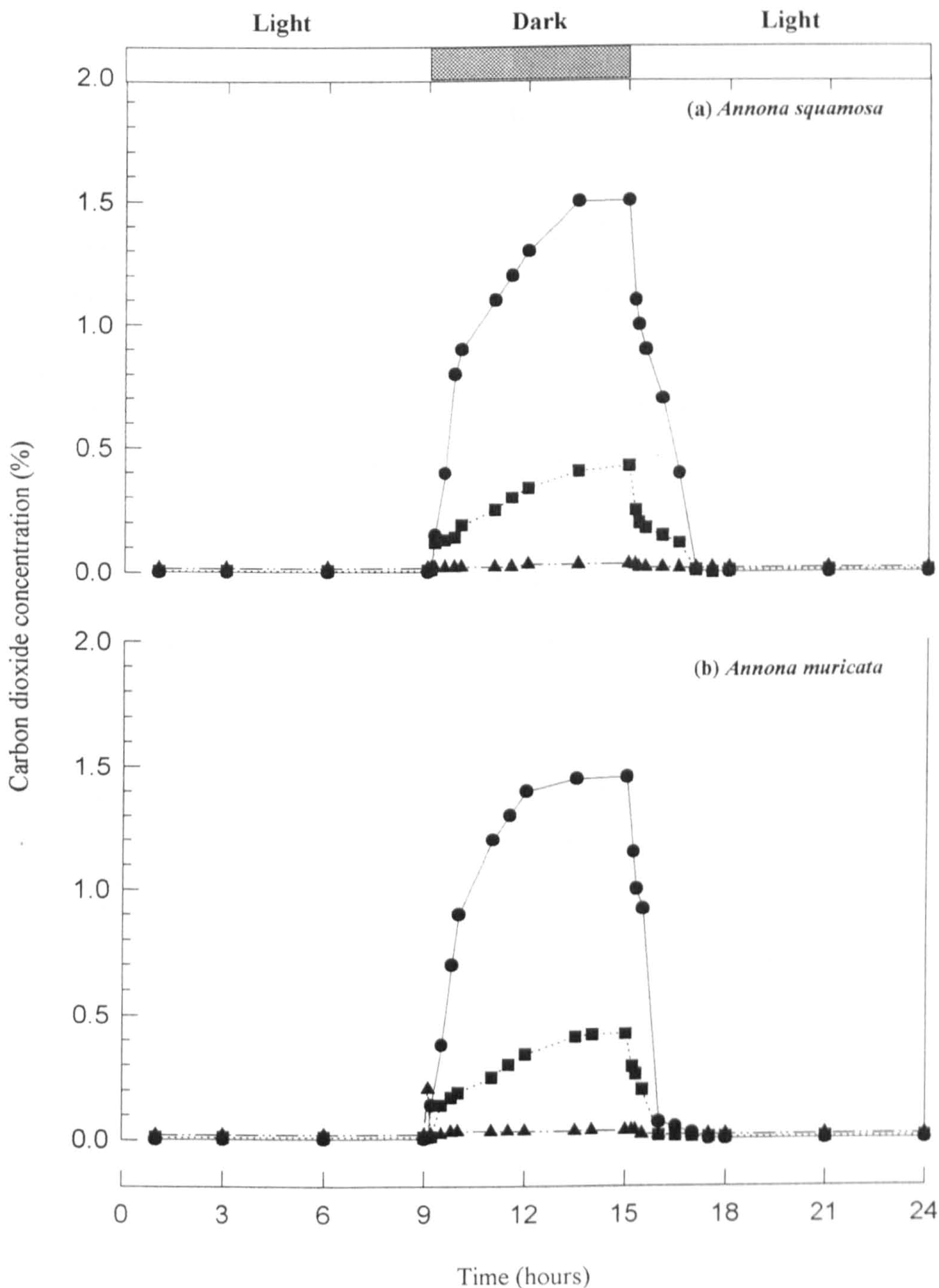



**Fig. 7.09.** Effects of different types of ventilation on $CO_2$ concentrations in the culture head-space of cuttings of (a) *Annona squamosa* L. (leaf area = 8.5 $cm^2$; total fresh weight = 423 mg) and (b) *Annona muricata* L. (leaf area = 7.9 $cm^2$; total fresh weight = 382 mg); 45 day old cultures were grown in 60 $cm^3$ vessels; Cultures were grown under continuous light at 80 $\mu mol\ m^{-2}\ s^{-1}$ (PAR), 25°C and 21% RH. Each vessel contained only one plantlet. Each symbol represents a mean ± SE of 5 replicates. ● = sealed with silicone rubber bung; ■ = capped with polypropylene disc (diffusive ventilation) and ▲ = capped with fast forced (FF) ventilation apparatus (flow rate = 5 $cm^3\ min^{-1}$).

With FF-ventilation in the light the $CO_2$ levels were sustained at even higher levels, 200 μl $l^{-1}$, than in the diffusive system despite an appreciably higher rate of consumption (i.e. higher photosynthesis rate: Fig. 7.05 and 7.06). The difference reflects the impedance to gas exchange of the polypropylene membrane in the diffusive system.

In the dark the $CO_2$ levels in the diffusive system did not rise as steeply as in the sealed vessels but attained levels of 2800 μl $l^{-1}$, and again reflecting the impedance to gas exchange of the polypropylene membrane. However, the more effective gas-exchange accompanied by FF-forced ventilation prevented the $CO_2$ concentrations from exceeding atmospheric levels (350 μl $l^{-1}$). Thus, night-time concentrations in the FF-system were 0.13X and 0.023X those in the diffusive and sealed systems respectively.

The findings described in this section and earlier suggest that in tightly sealed vessels low $CO_2$ concentrations will severely limit the photosynthetic rate and this should considerably hinder growth; it is likely also that this would eventually cause the death of plantlets. However, the sugar source in the supporting medium is probably sufficient to keep the plantlets viable considerably longer than has been the case in these experiments. Ethylene on the other hand severely depressed growth and caused senescence, and it must be concluded that the effects of endogenous ethylene accumulation in sealed vessels is a more immediate cause of senescence and death. Forced ventilation thus brings at least two major benefits: it prevents the accumulation of damaging quantities of ethylene and at the same time increases the supply of $CO_2$ which enhances photosynthetic rates (Figs 7.05 and 7.06) and stimulates growth (Tables 7.06 and 7.07). This will have positive feed-back properties and accentuate the benefits accruing from FF-ventilation. It is possible also that FF-ventilation may have a third advantage: that of preventing the build up of physiologically depressing levels of $CO_2$ during dark periods (Buddendorf-Joosten and Woltering 1994).

## 7.4. FINAL COMMENTS

Currently *Annona* spp. are propagated through grafting and budding, since for almost all of the species of *Annona* the clonal propagation by cuttings has not been very successful (Rasai, George and Kantharajah 1995). However, for rapid propagation and for the elimination of viral and other disease infections of horticultural species, tissue culture is an important tool (Frey 1981). Therefore, the micropropagation technique demonstrated in this chapter for *Annona* spp. may contribute towards their large scale propagation. However, further investigations are necessary to develop and improve the rooting systems of cuttings of both *Annona squamosa* and *A. muricata.*

In this study high rates of ethylene-induced leaf abscission were observed in both *Annona squamosa* and *A. muricata* plantlets/cuttings grown under conventional tissue culture systems (capped with polypropylene or sealed condition). Thus it was very difficult for the cuttings to become established. Similar results have also been found by Lemos and Blake(1994)and Armstrong *et al.* (1996) It was also revealed that *A. muricata* is more sensitive to ethylene, and higher rates of leaf abscission were recorded in this species. However, forced ventilation is an effective method for controlling leaf and flower-bud abscission and yield was also enhanced. Therefore, forced ventilation seems to be necessary for the effective flushing out of the ethylene from the culture vessels and for the establishment of *Annona* culture.

# CHAPTER - VIII

## EVALUATION OF CLOSED, DIFFUSIVE AND FORCED VENTILATION SYSTEMS ON GROWTH AND TUBERIZATION OF POTATO

### 8.1. INTRODUCTION

The propagation of potato *in vitro* by the serial culture of axillary shoots on separated nodes has been reported by a number of workers, and is now becoming established as an effective means of rapidly multiplying new or existing cultivars in disease-free conditions (Hussey and Stacey 1984). However, a major drawback to the procedure is that the potato plant is highly sensitive to ethylene, and ethylene accumulation *in vitro* strongly inhibits the growth and development of shoots. It is known that growth of potato plantlets can be distorted by concentrations of ethylene of 0.1 $\mu l\ l^{-1}$ or even less (Jackson *et al* 1987). Hussey and Stacey (1981) reported that in tightly closed culture vessels potato shoots become stoloniferous and leaves become small. Jackson *et al.* (1991) found that shoot height of *Solanum tuberosum* is reduced by 64% after 14 days of culture in tightly sealed vessels. They also concluded that accumulated ethylene is responsible for these effects. To remove ethylene from potato culture vessels, Jackson *et al* (1987) used mercuric perchlorate and thus increased shoot height. In this chapter, it was observed that the growth and development of root / shoot systems of potato (plantlets) was increased significantly by using $AgNO_3$ in the culture medium.

In recent years the *in vitro* tuberization phenomenon has become important for the rapid propagation of disease-free potatoes (Levy, Seabrook and Coleman 1993) and this has attracted the attention of a number of other researchers e.g. Akita and Takayama (1994) and Garner and Blake (1989). Miniature tubers (microtubers) formed on potato grown *in vitro* are useful also because they are very convenient for the maintenance and handling of disease-free material: microtubers are easily stored, transferred and distributed (Akita and Takayama 1994; Levy, Seabrook and Coleman 1993).

This chapter describes an *in vitro* tuberization technique, and an improvement in the culture conditions by the introduction of forced ventilation into the culture vessels

using the through-flow ventilation apparatus described in Chapter II and III. The chapter also describes investigations into the growth of this ethylene-sensitive and commercially important species with different types of ventilation and, in some cases, with and without the ethylene inhibitor silver nitrate or the ethylene precursor ACC. The aim of the experiments was to find ways of improving the culture conditions.

## 8.2. MATERIALS AND METHODS

### 8.2.1. Establishment of plantlets from tubers

Tubers of *Solanum tuberosum* L. 'cara' were washed clean with tap-water, cut into small pieces, approx. 15 $mm^3$, each containing a sprout, and were placed in paper bags inside an incubator at 21°C to allow rapid development of white etiolated sprouts which provided a source for the initial explants. These sprouts were sterilized with 10% v/v sodium hypochlorite solution, and cut into 1 cm nodal sections each containing a single axillary bud. For initial establishment and routine maintenance of cultures, these sections were inoculated on MS medium without any hormone. The cultures were kept at 23°C with a light flux of 100 $\mu mol\ m^{-2}\ s^{-1}$ (PAR) and a 16 h photoperiod Under these conditions a new shoot would develop from each node and at the four to five node stage these in turn were segmented into nodal sections to provide the experimental explant material.

Unless otherwise stated, establishment conditions of culture, culture vessels size and volume, media preparation, methods of measuring ethylene and $CO_2$ concentration etc., were as described in Chapter IV. The basal medium used was MS (Murashige and Skoog 1962).

### 8.2.2. Experiments

#### 8.2.2.1. Effects of the ethylene inhibitor ($AgNO_3$) on the growth of nodal stem cuttings

Single-node stem cuttings (F.W = *ca.* 40 mg and with one leaf) from potato plantlets grown *in vitro* were used as experimental material. The explants were subcultured on to MS medium containing silver at either 0.59, 2.96 or 5.9 $\mu mol\ l^{-1}$ ($AgNO_3$ = 0.1, 0.5 and 1.0 mg $l^{-1}$). At least five explants were inoculated for each treatment (one explant per vessel). The vessels were tightly sealed with silicone rubber bungs. Each vessel contained 10 ml of medium and 45 $cm^3$ of head-space. The cultures were incubated for 14 days under the same conditions described for the establishment of cultures. Growth measurements were performed after 14 days. These included total fresh weights of the

plants, leaf number and fresh weight, stem fresh weight and length and root number, fresh weight and maximum length.

#### 8.2.2.2. Effects of closed, diffusive and forced ventilation systems on growth and development with and without silver in the culture medium

Explants (nodal segments: mean fresh weights *ca.* 40 mg) were subcultured into glass vessels (as above) with the MS medium (10 ml) lacking any hormone, and six different treatments were given. These were:

(a) $AgNO_3$ was added in the medium to a concentration of 2.96 μmol $l^{-1}$ (0 5 $mgl^{-1}$) and the vessels were sealed with silicone rubber bungs.

(b) No $AgNO_3$ was added and the vessels were sealed with silicone rubber bungs.

(c) No $AgNO_3$ was added and the vessels were capped with polypropylene membranes.

(d) $AgNO_3$ was added to a concentration of 2.96 μmol $l^{-1}$ and the vessels were capped with polypropylene membranes.

(e) No $AgNO_3$ was added and each vessel was fitted with a fast flow (FF) convective flow unit (flow rate *ca.* 3.5 $cm^3$ $min^{-1}$, see Chapter II).

(f) No $AgNO_3$ was added and each vessel was fitted with a slow flow (SF) convective flow unit (flow rate *ca.* 1.0 $cm^3$ $min^{-1}$).

Five vessels were prepared for each treatment (one explant per vessel) and the cultures were incubated for 18 days under continuous light in the same conditions described for the establishment of cultures. Harvesting was performed on the 18th day and growth measurements were as in Section 8.2.2.

**8.2.2.3. Effects of ventilation types and the ethylene inhibitor ($AgNO_3$) and the ethylene precursor (ACC) on the growth of nodal stem cuttings.**

Nodal stem cuttings were inoculated into the culture vessels as described in Section 8.2.2, and grown under the different treatments described below, where, in these experiments the FF-system delivered a flow rate of *ca.* 5 $cm^3$ $min^{-1}$:

(a) vessels were sealed with silicone rubber bungs,

(b) vessels sealed with silicone rubber bungs and $AgNO_3$ in the medium at 2.96 $\mu mol\ l^{-1}$,

(c) sealed with silicone rubber bung and ACC in the medium at 2.0 $\mu mol\ l^{-1}$,

(d) vessels capped with polypropylene membrane,

(e) vessels capped with polypropylene membrane and $AgNO_3$ in the medium at 2.96 $\mu mol\ l^{-1}$,

(f) vessels capped with polypropylene membrane and ACC in the medium at 2.0 $\mu mol\ l^{-1}$,

(g) fitted with FF-ventilation apparatus,

(h) fitted with FF-ventilation apparatus and $AgNO_3$ in the medium at 2.96 $\mu mol\ l^{-1}$,

(i) fitted with FF-ventilation apparatus and ACC in the medium at 2.0 $\mu mol\ l^{-1}$.

Carbon dioxide, oxygen, ethylene and relative humidities were measured at certain intervals over the first 21 days of the experiment. In contrast to the previous experiment the plants were grown under continuous illumination as for the tocacco and cauliflower described in Chapters IV and V. Plants were harvested after 25 days. Growth measurements included leaf number, area and fresh weight, stem fresh weight and length, root number and maximum length and volume of callus.

**8.2.2.4. Tuberization *in vitro***

To ascertain the best concentrations of sucrose and BAP for tunerization, explants (nodal segments) were inoculated in 60 $cm^3$ culture vessels on MS medium supplemented with BAP (0.0 - 2.0 mg $l^{-1}$) and different sucrose concentrations (4%, 8%,

and 12%). The cultures were incubated under growth room conditions with a 16h photoperiod.

Having determined the optimum concentrations of sucrose and BAP for tuberization the different ventilation treatments were tested. The cultures were grown on MS medium supplemented with BAP at 1.0 mg $l^{-1}$ and 8% sucrose, for optimum growth, and replicates (five per treatment) were cultured for 6-8 weeks, each vessel fitted with either (a) a silicone rubber bung (b) a polypropylene membrane (c) a FF-ventilation apparatus (5.0 $cm^3$ $min^{-1}$) and (d) a SF-ventilation apparatus (1.0 $cm^3$ $min^{-1}$). Cultures were inoculated under growth room conditions with a 16h photoperiod. .Plants were harvested after 8 weeks; growth measurements included fresh weights and numbers of tubers.

## 8.3. RESULTS AND DISCUSSION

### 8.3.1. Effects of the ethylene inhibitor ($AgNO_3$) under sealed conditions

Silver nitrate at 0.59 and 2.96 µmol $l^{-1}$ in the medium had a significant stimulatory effect on the growth and development of the potato shoots *per se* (Table 8.01; Plate 8.01): the increases in fresh weights in these treatments were substantially greater than those in the control (Table 8.01). On the other hand 5.9 µM silver seemed to have marginally depressed shoot growth. Again, the best root systems were developed in the 0.59 and 2.96 µmol $l^{-1}$ treatments: fresh weights were *ca.* 2-3 times greater than the control (Table 8.01). Also, root growth was depressed by 5.9 µM silver.

**TABLE 8.01.** **Effects of ethylene inhibitor ($AgNO_3$) on the growth and development of potato (*Solanum tuberosum* L.) stem cuttings after 14 days in sealed vessels; 60 $cm^3$ culture vessels were sealed with silicone rubber bungs. Each value represents a mean ± SE of 5 replicates.**

| Characteristics | Control | $AgNO_3$ (0.59 µM) | $AgNO_3$ (2.96 µM) | $AgNO_3$ (5.9 µM) |
|---|---|---|---|---|
| **Leaves** | | | | |
| Number | 4.0±1.0 | 6.0±1.0 | 16.7±1.2 | 6.5±0.6 |
| Fresh weight (mg) | 0.96±0.1 | 2.1±0.5 | 4.0±1.3 | 1.6±0.3 |
| **Shoots** | | | | |
| Length (mm) | 14.4±5.0 | 47.2±7.4 | 32.5±4.9 | 9.6±0.3 |
| *Fresh weight (mg) | 5.9±1.9 | 15.9±6.3 | 18.0±2.3 | 3.6±0.2 |
| **Roots** | | | | |
| †Length (mm) | 21.4±5.2 | 35.2±4.9 | 35.3±6.9 | 16.2±0.8 |
| Number | 3.3±0.9 | 6.3±1.2 | 7.3±0.6 | 3.3±0.6 |
| Fresh weight (mg) | 2.8 ±0.2 | 7.6±0.7 | 9.5±2.8 | 2.1±1.4 |
| *Total Fresh weight (mg) | 9.8±2.8 | 29.6±11.5 | 31.7±6.6 | 6.7±0.8 |

* increased fresh weight; † Mean maximum root length (10 roots).

The clearest difference to emerge between the 0.59 and 2.96 µmol $l^{-1}$ treatments was in the numbers of leaves produced: leaf numbers in the 2.96 µmol $l^{-1}$ treatment were more than double those at the lower of these concentrations. Leaf area was also greatest in this treatment compared with the others (data not shown). The highest shoot length was

**PLATE : 8.01**

Potato (*Solanum tuberosum* L.) plantlets obtained from stem cuttings and cultured in MS medium supplemented with different concentrations of the ethylene inhibitor ($AgNO_3$); 60 $cm^3$ culture vessels were sealed with silicone rubber bungs. Note stimulation of growth by $AgNO_3$ at 0.1 $mgl^{-1}$ (0.59 µM) and 0.5 $mgl^{-1}$(2.96 µM), but slight inhibition at 1.0 $mgl^{-1}$ (5.9 µM) (X0.8).

CONTROL
AgNO3 (1.0 mgl -1)
AgNO3 (0.5 mgl -1)
AgNO3 (0.1 mgl -1)

recorded in the 0.59 µmol $l^{-1}$ $AgNO_3$ treatments but here the shoots were noticeably narrower than in the 2.96 µmol $^{-1}$ treatment.

After studying all the parameters of growth and development it was concluded that the potato plantlets showed their best performance at a $AgNO_3$ concentration of 2.96µmol $l^{-1}$ (0.5 mg $l^{-1}$). At the highest silver concentration growth was generally depressed below that of the control (Plate 8.01).

By using mercuric perchlorate to remove ethylene from the potato culture vessel Jackson *et al* (1987) were able to improve shoot height (72 mm compared to 62 mm in the control). In the above experiment, by using $AgNO_3$ (0.59 µmol $l^{-1}$ = 0.1 $mgl^{-1}$) shoot height was 2-3 times greater than the untreated control. Jackson *et al* (1987) also pointed out that when potato shoots are grown in tightly sealed culture vessels, stems become swollen, leaves become small and root systems are very poor. Similarly, in the present experiment in the control, shoots were short, and stems became very thick with tiny leaves. These are said to be typical of the effects of ethylene.

### 8.3.2. Effects of closed, diffusive and forced ventilation systems and the ethylene inhibitor, silver nitrate, on growth and development

In this investigation, after studying all the parameters of growth and development within the various treatments, it is evident that explants tended to grow better with forced-ventilation (Table 8.02; Plate 8.02). After 18 days of culture, the best leaf, stem and root growth were observed in explants grown with the more rapid convective ventilation (3 5 $cm^3$ $min^{-1}$ flow rate); the values of most parameters were higher even than those with diffusive ventilation plus silver. In a number of respects the effects of SF-forced ventilation were also superior to those of diffusive-flow e.g. in terms of root numbers, leaf numbers and fresh weight, and stem lengths. However, with the addition of silver in the diffusive flow treatment, shoot fresh weights were similar to those of the SF-treatment and root lengths and fresh weights were greater.

## PLATE : 8.02

The influence of different methods of capping of culture tubes on shoot culture of potato (*Solanum tuberosum* L.) plantlets (18 days old); cultures were kept at 23°C with a light flux of 100 µmol $m^{-2}$ $s^{-1}$ (PAR) and a 16 h photoperiod (X0.7).

The treatments are :

(A) $AgNO_3$ added (2.96 µmol $l^{-1}$ : 0.5 $mgl^{-1}$) and the vessels were sealed with silicone rubber bung.

(B) No $AgNO_3$ added and the vessels were sealed with silicone rubber bungs.

(C) No $AgNO_3$ added and the vessels were capped with polypropylene membrane.

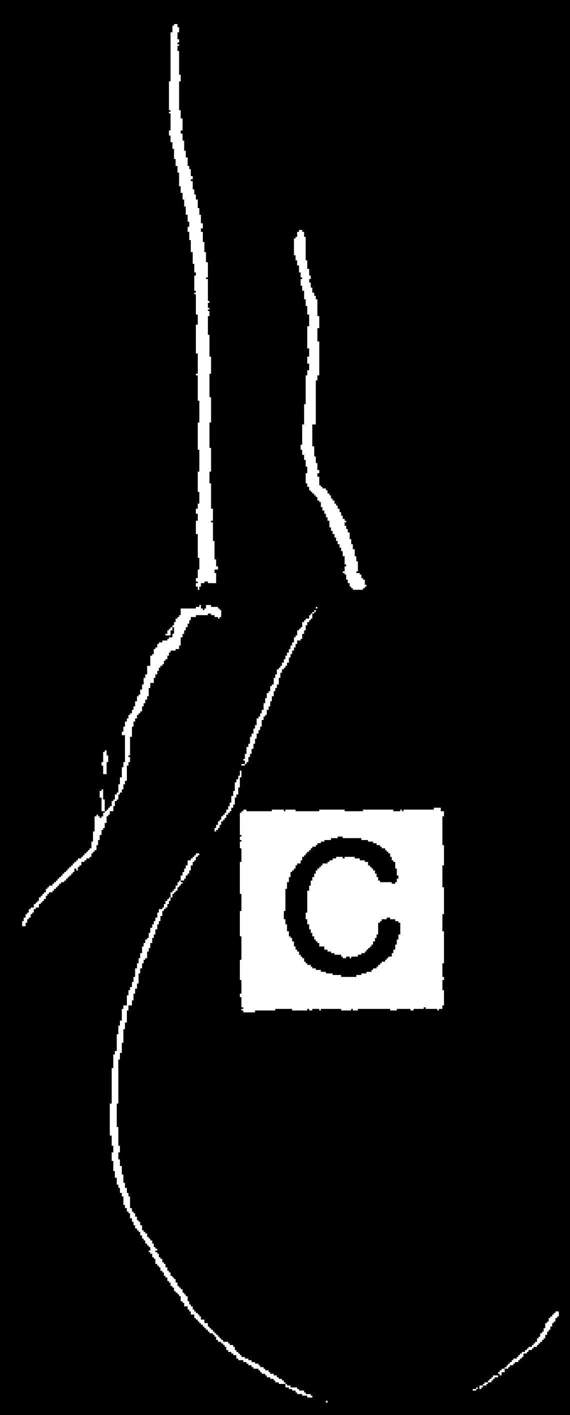
(D) $AgNO_3$ added (2.96 µmol $l^{-1}$ · 0.5 $mgl^{-1}$) and the vessels capped with polypropylene membrane.

(E) No $AgNO_3$ added and each vessel was fitted with a fast flow (FF) convective flow unit (flow rate *ca.* 3.5 $cm^3$ $min^{-1}$).

(F) No $AgNO_3$ added and each vessel was fitted with a slow flow (SF) convective flow apparatus (flow rate *ca.* 1.0 $cm^3 min^{-1}$).

Note poor growth in sealed condition and with diffusive ventilation, but in each case $AgNO_3$ stimulated growth. However, in the absence of $AgNO_3$ both fast and slow flow ventilations stimulated the best growth.

A

B

C

D

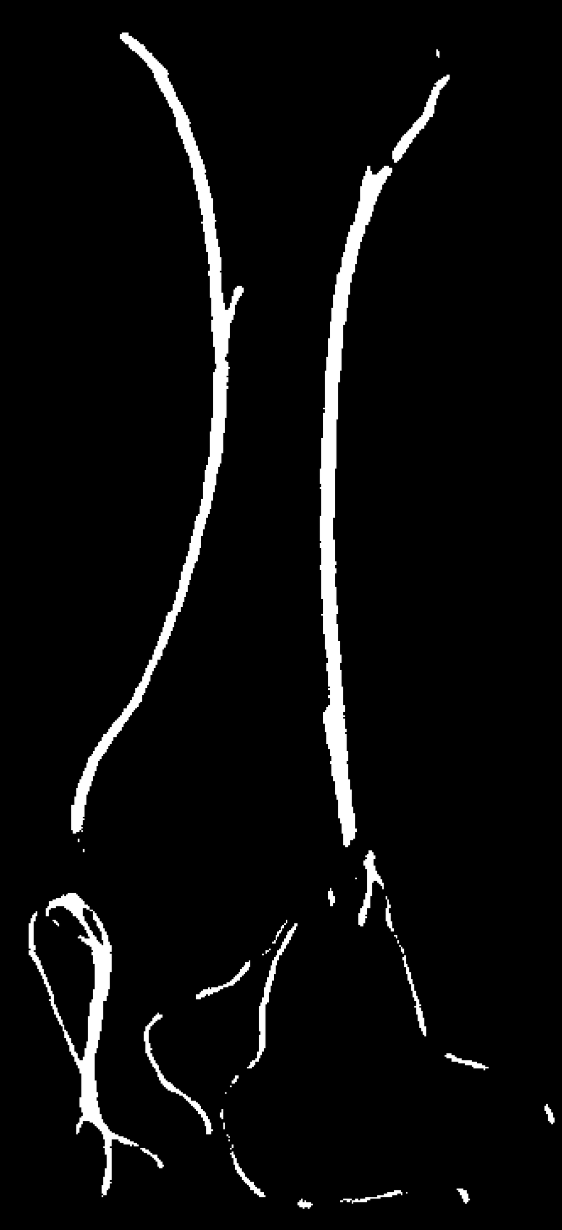
E

F

**TABLE 8.02.** **Effects of closed, diffusive and forced ventilation systems on growth and development of *in vitro*-grown potato (*Solanum tuberosum* L.) plantlets (18 days old); cultures were kept at 23°C with a light flux of 100 mmol $m^{-2}$ $s^{-1}$ (PAR) and a 16 h photoperiod. Each value represents a mean ± SE of 5 replicates.**

| Morphology | Sealed | Sealed + $AgNO_3$ (2.96 µM) | Diffusive ventilation | Diffusive ventilation + $AgNO_3$ (2.96 µM) | Forced ventilation (flow rate = 1.0 $cm^3 min^{-1}$) | Forced ventilation (flow rate = 3.5 $cm^3 min^{-1}$) |
|---|---|---|---|---|---|---|
| **Leaves** | | | | | | |
| Number | 2.7±0.6 | 6.0±1.0 | 5.6±0.6 | 6.3±0.6 | 9.0±1.0 | 10.3±2.1 |
| FW (mg) | 0.6±0.01 | 5.8±0.3 | 6.5±0.4 | 6.8±0.2 | 12.1±1.4 | 17.2±1.1 |
| **Shoots** | | | | | | |
| Length (mm) | 30.7±1.5 | 32.3±0.1 | 37.7±7.0 | 51.6±3.2 | 58.5±3.8 | 73.8±6.1 |
| *FW (mg) | 27.5±0.4 | 34.1±0.4 | 37.5±0.5 | 40.9±0.2 | 39.9±6.4 | 53.7±9.3 |
| **Roots** | | | | | | |
| †Length (mm) | 35.4±3.5 | 33.7±1.9 | 26.6±4.6 | 42.9±5.9 | 30.2±7.5 | 61.3±11.6 |
| Number | 3.7±0.6 | 5.0±1.0 | 4.3±0.9 | 4.7±0.6 | 8.0±2.7 | 9.0±1.0 |
| FW (mg) | 7.6±0.5 | 10.4±1.2 | 7.5±0.5 | 16.3±0.2 | 10.0±1.3 | 17.3±0.7 |

* increased fresh weight; †Mean maximum root length (10 roots).

The suppression of ethylene activity by silver was also very evident elsewhere: in the sealed condition the addition of silver led to a doubling of the leaf number, while leaf fresh weight increased six-fold and the fresh weight of the roots also increased. In the case of diffusive ventilation the addition of silver increased root and shoot lengths and approximately doubled the root fresh weight; other parameters showed a tendency to increase but in terms of significance only marginally.

When plantlets were grown in the tightly sealed condition, shoots were short (*ca.* 31 mm in length) swollen and the leaves small with a tendency to be folded. Stem apices became hooked in shape, and root systems were stunted. Some shoots became brown at the tips. These results are consistent with earlier observations of Jackson *et al* (1987) and Hussey and Stacey (1984) and associated with the effects of ethylene. In contrast, plantlets grown under forced ventilation had the best developed shoot and root systems, and morphologically the plants were normal with normal stem apices (Plate 8.02).

Jackson *et al* (1991) acknowledged that the problem of ethylene accumulation can be lessened by the use of larger culture vessels. However, the system described here would enable the use of smaller vessels. A further possible advantage of forced ventilation is that the aerating gases are humidified, and this should help to reduce losses of water vapour from both plants and medium.

It is likely that with longer-term growth under micropropagation the differences found in this experiment would become even more accentuated. for example it is probable that $CO_2$ levels will have been nearer to the compensation point in the diffusive flow system than in the forced flow systems (see Chapters IV and V). Consequently photosynthetic rates in the forced-flow systems will have been greater and the positive feed-back effects of this might well be cumulative beyond the 18-day growth period adopted here.

### 8.3.3. Effects of ventilation types and the ethylene inhibitor ($AgNO_3$) and the ethylene precursor (ACC) on the growth of nodal stem cuttings.

#### 8.3.3.1. Growth

As in the previous experiment the potato responded very favourably to forced ventilation and the differences between treatments and the effects of silver additions also were very similar. In the present experiment, however, there were also ACC additions to some treatments and it is interesting to note that despite a stimulation of ethylene production (see Fig. 8.01 and below) the ACC did not, within ventilation treatments, noticeably reduce further the growth of leaves or shoots. On the other hand ACC additions very much reduced root growth; this may have been because the roots were the major site of ethylene production and, because the roots were embedded in agar, its escape would be hindered. Thus, endogenous concentrations might have reached very inhibitory levels. Silver nitrate alleviated these effects (Table 8.03).

The higher shoot fresh weights found in the additive-free sealed controls and in the sealed + ACC systems compared with their diffusive counterparts may be accounted for by ethylene-induced swelling of the shoots.

**Table 8.03.** Effects of different types of ventilation on growth and development of *in vitro*-grown potato (*Solanum tuberosum* L.) stem cuttings (25 days old); cultures were kept at 25°C with a light flux of 150 µmol $m^{-2}$ $s^{-1}$ (PAR) and a 24 h photoperiod.

| Treatments | Methods of ventilation | Leaf | | | Stem | | Root | | Callus |
|---|---|---|---|---|---|---|---|---|---|
| | | F.W. (mg) | Number | Area ($cm^2$) | *F.W (mg) | Length (mm) | †Length (mm) | Number | Vol ($cm^2$) |
| **Control** | Sealed | 0.9±0.2 | 4.6±0.8 | 0.6±0.1 | 38.1±5.3 | 31.3±4.3 | 20.7±3.1 | 12.8±3.4 | 1.9±0.3 |
| | Diffusive ventilation | 7.5±1.3 | 5.3±0.5 | 3.0±0.3 | 27.8±4.1 | 36.3±4.2 | 55.1±4.1 | 10.5±3.9 | 0.6±0.1 |
| | Forced ventilation (Fast) | 19.5±1.2 | 8.2±0.9 | 4.7±1.1 | 42.5±4.4 | 41.8±5.1 | 95.2±9.2 | 12.3±3.5 | - |
| **ACC** | Sealed | 1.2±0.3 | 4.9±0.8 | 0.7±0.1 | 40.2±4.4 | 32.4±5.2 | 11.4±2.1 | 17.3±5.5 | 2.7±0.2 |
| (2.0 µM) | Diffusive ventilation | 5.3±1.2 | 5.1±0.3 | 2.1±0.5 | 25.8±3.5 | 29.7±3.1 | 13.1±2.2 | 13.9±2.0 | 0.9±0.3 |
| | Forced ventilation (Fast) | 20.0±1.8 | 7.9±0.8 | 4.8±1.4 | 45.5±3.3 | 40.9±2.0 | 101.0±10 | 12.5±3.3 | - |
| **$AgNO_3$** | Sealed | 6.1±1.1 | 5.0±0.5 | 2.9±0.4 | 21.2±3.1 | 32.1±4.1 | 88.9±8.1 | 7.6±2.8 | - |
| (2.95 µM) | Diffusive ventilation | 10.8±4.2 | 6.3±0.6 | 4.4±0.6 | 35.5±4.6 | 34.6±3.7 | 99.9±10 | 7.7±9.9 | - |
| | Forced ventilation (Fast) | 21.7±8.5 | 6.9±0.4 | 5.0±0.9 | 46.5±4.1 | 41.5±3.8 | 110.9±15 | 12.2±4.7 | - |

* callus fresh weight were not included; †Mean maximum root length (10 roots).
*fast flow ventilation rate = 3.5 $cm^3$ $min^{-1}$ ; for sealed and diffusive ventilation vessels were capped with silicone rubber bungs and polypropylene discs respectively.

A major effect noted in this experiment was the development of callus in the sealed and diffusive treatments, with or without the addition of ACC (Plate 8.03); however, ACC appeared to increase the quantity of callus produced. Silver prevented callus induction, as did forced-flow ventilation. It should be noted that, where (as in this case) the culture medium has not been designed to stimulate callus development, its production is commonly associated with vitrification (Paque and Boxus, 1987; Ziv, 1991b). None of the plants here had brittle leaves but they did develop hooked shoot tips which is another symptom associated with vitrification (Jackson *et al.* 1987). Again both silver and forced-flow ventilation prevented the formation of hooked tips.

**8.3.3.2. Head-space atmosphere**

**Ethylene:**

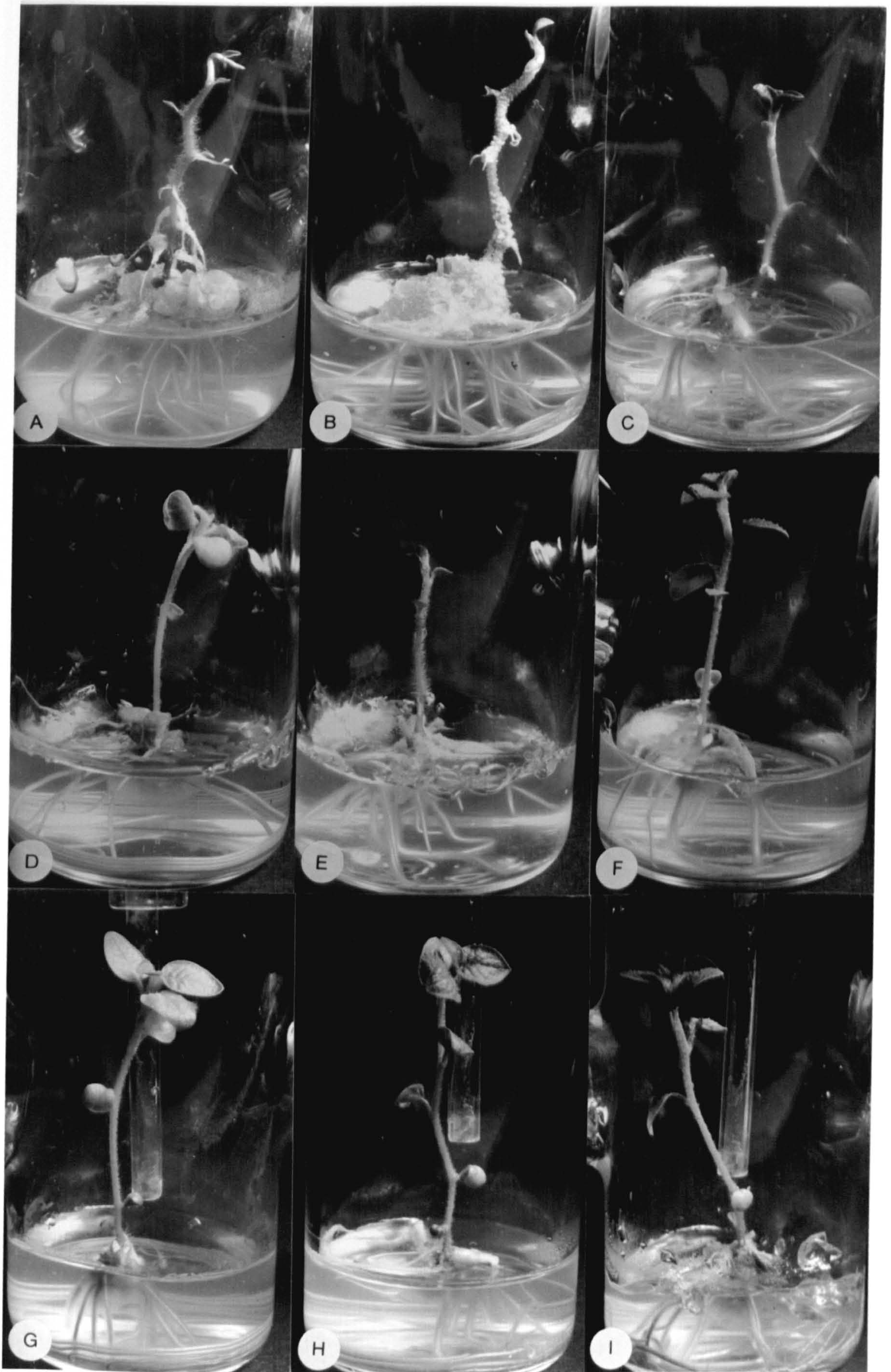
In sealed vessels, the addition of ACC in the medium resulted in very high concentrations of ethylene: after only 12 days 1.45 $\mu l\ l^{-1}$ had accumulated and this was 2.3X that of the sealed control (Fig. 8.01). Subsequently the ethylene levels in the ACC treatment fell back while in the sealed controls they continued to rise so that by 21 days the differences between the two were much smaller than before. In the sealed silver treatment the ethylene concentration was higher than that of the sealed control; it is presumed that this was a function of the much larger plant size in the silver treatment. Diffusive ventilation resulted in much lower ethylene accumulation (0.032 $\mu l\ l^{-1}$ in control and 0.086 $\mu l\ l^{-1}$ in the silver treatment), although the ACC addition produced significantly higher levels (0.41 $\mu l\ l^{-1}$). Unfortunately due to the wide variability it is not possible to deduce from the effects of silver addition whether any of these concentrations in the diffusive systems were physiologically active. Nevertheless it is clear that the polypropylene membranes helped reduce ethylene accumulation markedly. On the other hand, forced ventilation was even more effective at minimising ethylene accumulation and the gas was virtually undetectable even in the ACC treatments.

## PLATE : 8.03

The influence of different methods of capping of culture tubes on shoots culture of potato (*Solanum tuberosum* L.) plantlets (25 days old); cultures were kept at 25°C with a light flux of 150 μmol $m^{-2}$ $s^{-1}$ (PAR) and a 24 h photoperiod (X1.3).

(A) vessels were sealed with silicone rubber bungs.
(B) sealed with silicone rubber bungs and 2.0 μM ACC in the medium.
(C) vessels sealed with silicone rubber bungs and 2.96 μM $AgNO_3$ in the medium.
(D) vessels capped with polypropylene membranes.
(E) vessels capped with polypropylene membranes and 2.0 μM ACC in the medium.
(F) vessels capped with polypropylene membranes and 2.96 μM $AgNO_3$ in the medium.
(G) fitted with FF-ventilation apparatus.
(H) fitted with FF-ventilation apparatus and 2.0 μM ACC in the medium.
(I) fitted with FF-ventilation apparatus and 2.96 μM $AgNO_3$ in the medium.

Note that (1) in absence of additives, A, D, G, growth and leaf expansion improved greatly, but callus growth decreased with increased ventilation; callus growth is a sign of vitrification; (2) in sealed and diffusive treatments B, E, ACC stimulated growth of callus and root numbers and in the latter reduced leaf area; (3) $AgNO_3$ stimulated growth in sealed and diffusive treatments C, F; and (4) with forced ventilation growth was best in all treatments; neither ACC nor $AgNO_3$ had any large effects and there was no callus growth; (5) in the sealed treatment only, ± ACC the shoot tips were hooked (a sign of vitrification), but $AgNO_3$ produced normal apical growth.


A
B
C
D
E
F
G
H
I

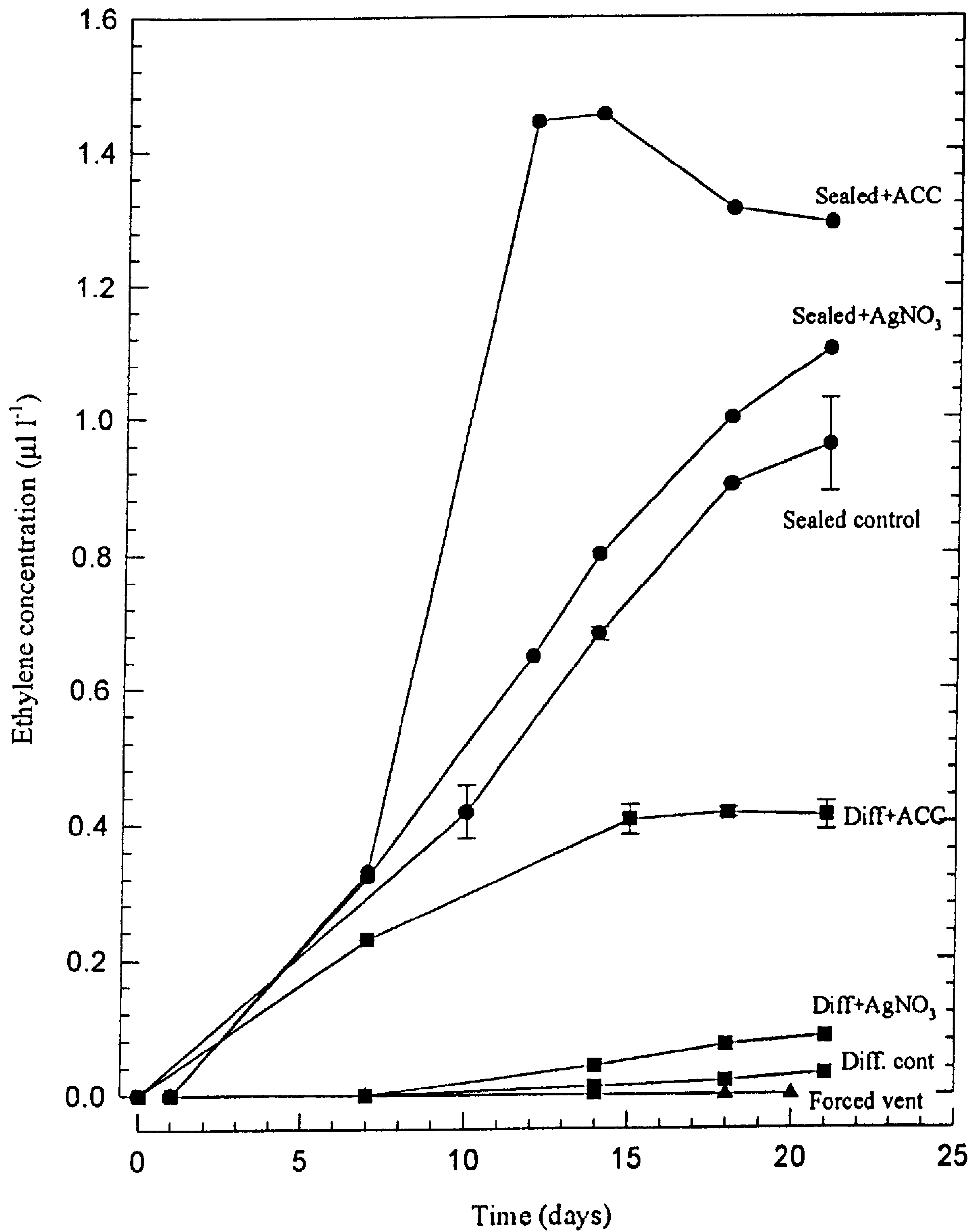



**Fig. 8.01.** Effects of different types of ventilation and ACC (2 μM) and $AgNO_3$ (2.96 μM) on ethylene concentrations in the head-space above potato cultures in 60 $cm^3$ vessels; cultures were grown under continuous light at 150 μmol $m^{-2}$ $s^{-1}$ (PAR), 25°C and 31% RH. Each symbol represents a mean ± SE of 5 replicates. Sealed = sealed with silicone rubber bung; diff. = diffusive ventilation; forced ventilation rate = 5 $cm^3$ $min^{-1}$.

**Oxygen**:

In terms of the temporal patterns in head-space oxygen regime, the results in Fig. 8.02 reveal very distinct differences between forced ventilation and the other two ventilating systems. Thus, with each of the forced-flow treatments concentrations remained constant and close to atmospheric for the whole period, whereas with diffusive and sealed ventilation they declined at varying rates from a little above atmospheric. The initial concentrations presumably reflected some photosynthetic enhancement of oxygen within the head-space. Also, within the diffusive and sealed treatments, and presumably due to their effects on the plants, silver nitrate or ACC additions can be seen to have influenced the rate of decline in the oxygen levels.

In the sealed-control and -ACC treatments, the oxygen concentrations fell substantially during the experiment: after 21 days of culture there were respectively only 14.8% and 11.6% oxygen in the head-spaces compared to *ca.* 20% in the equivalent forced ventilation treatments (Fig. 8.02). With $AgNO_3$ in the culture medium the oxygen concentration in the head-space of the sealed vessels was very much higher than that of ACC or control treatments and only a little lower than treatments having forced ventilation.

The diffusively ventilated treatments showed a similar pattern to their sealed counterparts but the differences were less. Thus, in the ACC treatment the oxygen concentration had dropped to approx. 16% over 21 days, while in the control and silver treatments the values were *ca.* 17.5% and 19.5% respectively.

In view of the data obtained with cauliflower (Section 4.3.5.3), and the growth parameters recorded in Table 8.03 and Plate 8.03, it seems likely that the gradual depression in the oxygen concentrations in the sealed and diffusive treatments lacking silver will have been due to (a) increased respiratory demands associated with the production of varying quantities of non-photosynthetic callus, and the development of the root systems, and possibly (b) to some degree of senescence affecting the photosynthetic tissues.

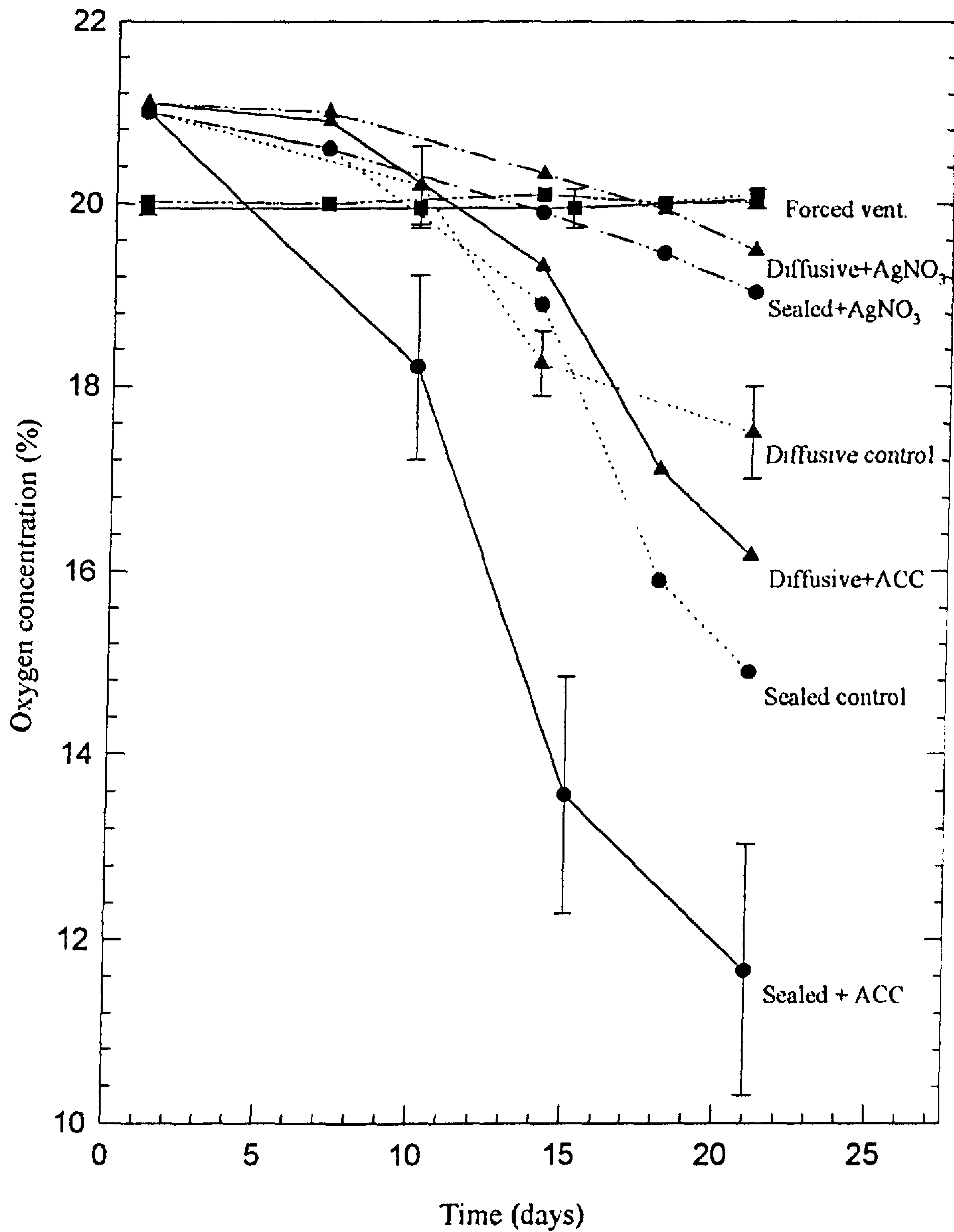



**Fig. 8.02.** Effects of different types of ventilation and ACC (2 μM) and $AgNO_3$ (2.96 μM) on oxygen concentrations in the head-space above potato cultures in 60 $cm^3$ vessels; cultures were grown under continuous light at 150 μmol $m^{-2}$ $s^{-1}$ (PAR), 25°C and 31% RH. Each symbol represents a mean ± SE of 5 replicates. Sealed = sealed with silicone rubber bung; diff. = diffusive ventilation; forced ventilation rate = 5 $cm^3$ $min^{-1}$.

These results are consistent with the findings of some other authors. In tightly sealed vessels with *Ficus* plantlets, oxygen concentrations of approximately 10% were observed (Jackson *et al.*, 1991). In a sealed petri-dish with rice callus the oxygen concentration was 2 to 5% after 24 days of culture (Adkins, Shiraishi and McComb 1990).

**Carbon dioxide:**

On a scale of zero to 4% (Fig. 8.03a) changes in carbon dioxide concentration are barely noticeable until day 14 by which time the level in the sealed control plus ACC had reached 0.9%; by day 21 the concentration was nearly 4%. The effect here and in the sealed control and diffusive treatments with and without ACC are probably attributable to the respiratory activity of the callus which developed. Thus, the balance between photosynthesis and respiration was moved in favour of respiratory $CO_2$ output.

In the other treatments: forced ventilation controls and forced-, diffusive- and sealed-ventilation with silver nitrate addition, callus did not form and $CO_2$ levels remained relatively constant or declined with time (Fig. 8.03b) with the decline being greatest where ventilation was poorest. Thus, in the sealed controls + $AgNO_3$, $CO_2$ levels were at or close to the compensation point (45 $\mu l\ l^{-1}$) by 21 days. Diffusive ventilation improved this position and after 21 days there was still a $CO_2$ concentration of 200 $\mu l\ l^{-1}$ in silver treatment. In all the forced-ventilation treatments the $CO_2$ concentrations remained above 300 $\mu l\ l^{-1}$ despite the greater $CO_2$ demand associated with the greater productivity.

Again, therefore, the results confirm the benefits of forced ventilation.

### 8.3.4. Development of an *in vitro* tuberization technique for potato

Shoots were grown in MS medium with different hormonal treatments to develop an ideal tuberization medium. In the control (without any hormone) no tuberization was observed but shoot systems were well developed. When a low concentration of BAP (0.5 mg $l^{-1}$) was present in the medium, the percentage of tuberization was very low. The best tuberization was observed in medium containing 1.0 mg $l^{-1}$ BAP (Table 8.04; Plate 8.04).

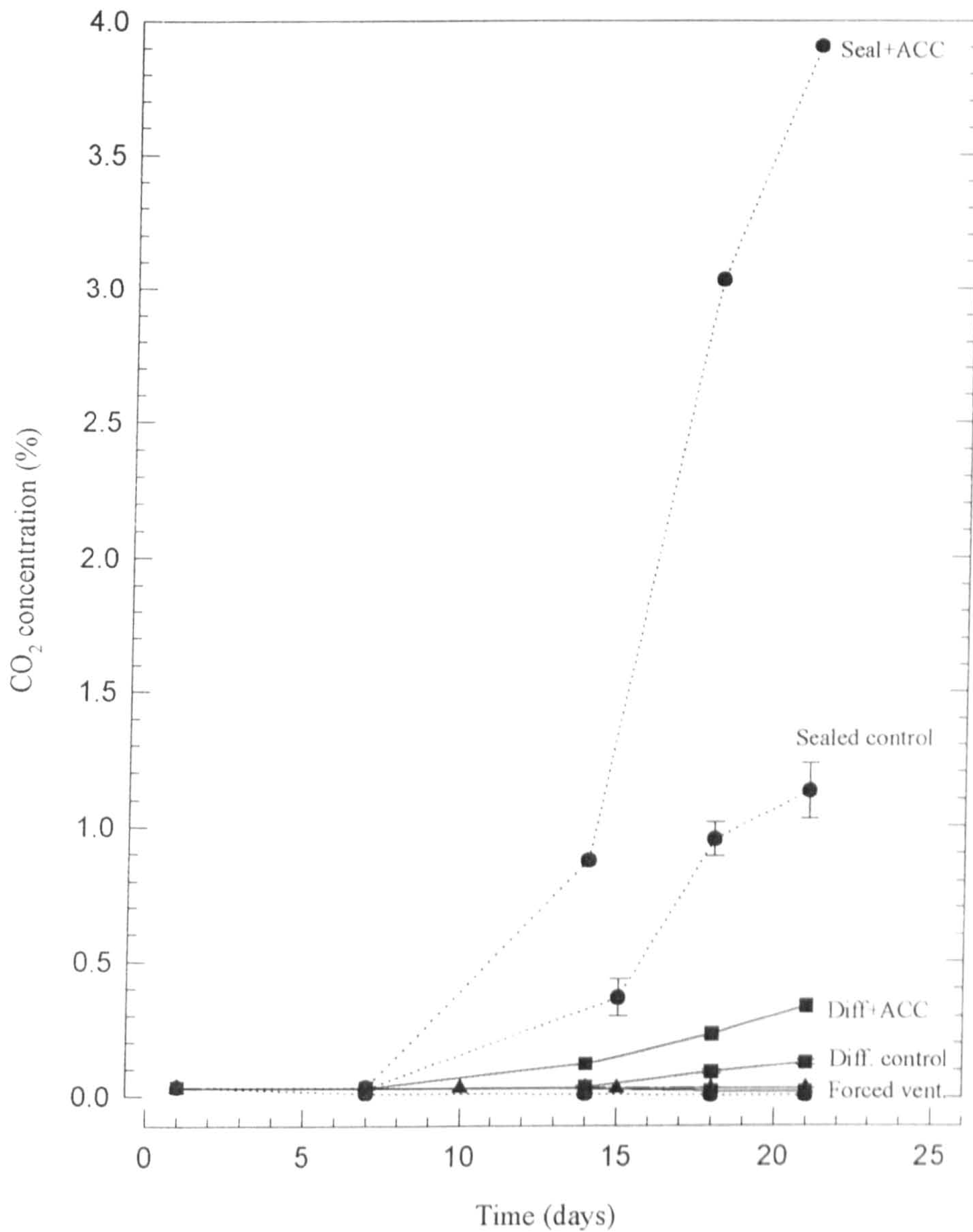



**Fig. 8.03a.** Effects of different types of ventilation and ACC (2 µM) and $AgNO_3$ (2.96 µM) on carbon dioxide concentrations in the head-space above potato cultures in 60 $cm^3$ vessels; cultures were grown under continuous light at 150 µmol $m^{-2}$ $s^{-1}$ (PAR), 25°C and 31% RH. Each symbol represents a mean ± SE of 5 replicates. Sealed = sealed with silicone rubber bung; diff. = diffusive ventilation; forced ventilation rate = 5 $cm^3$ $min^{-1}$. $CO_2$ on scale of zero to 4%.

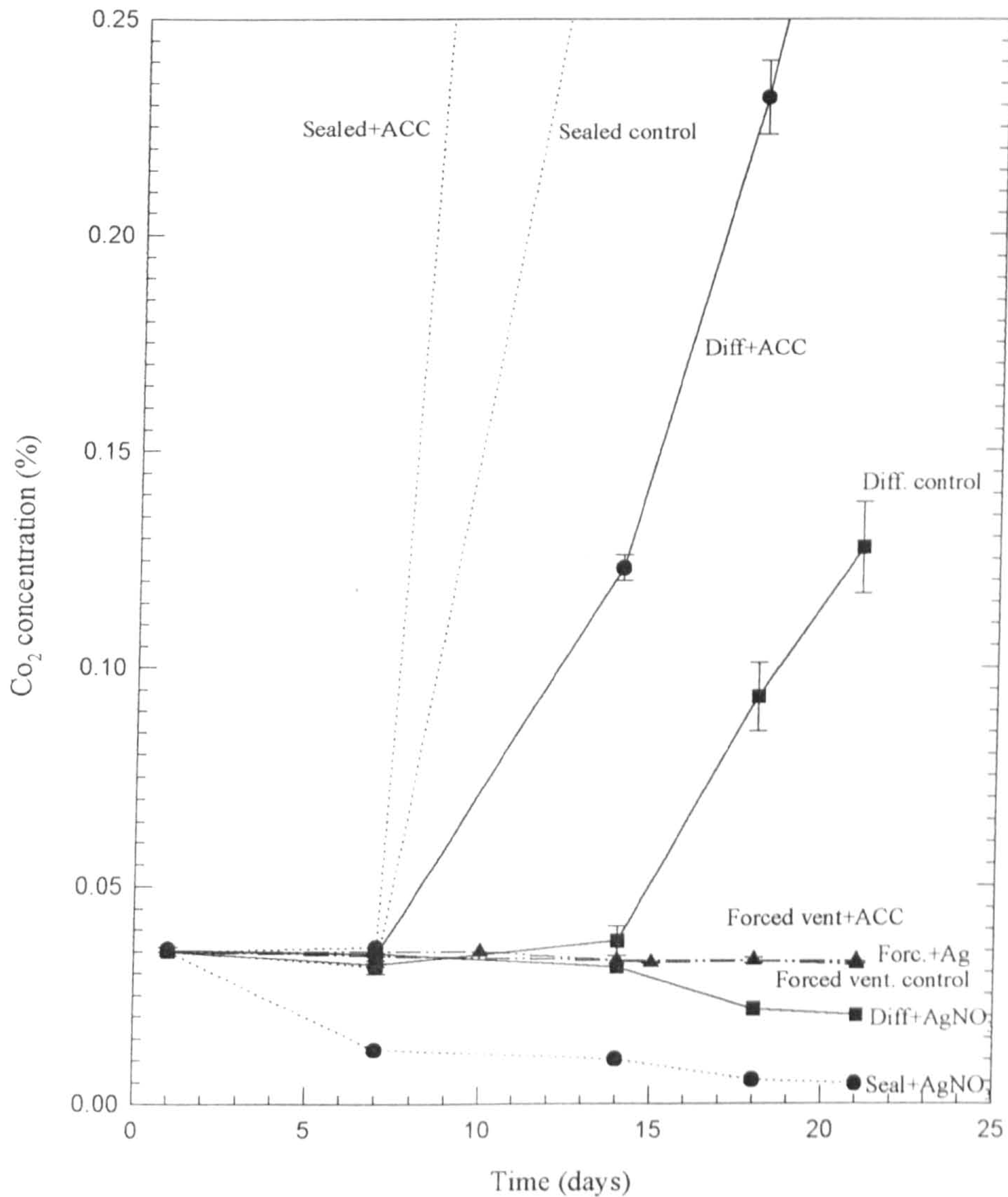



**Fig. 8.03b.** Effects of different types of ventilation and ACC (2 µM) and $AgNO_3$ (2.96 µM) on carbon dioxide concentrations in the head-space above potato cultures in 60 $cm^3$ vessels; cultures were grown under continuous light at 150 µmol $m^{-2}$ $s^{-1}$ (PAR), 25°C and 31% RH. Each symbol represents a mean ± SE of 5 replicates. Sealed = sealed with silicone rubber bung; diff.=diffusive ventilation; forced ventilation rate=5 $cm^3$ $min^{-1}$. $CO_2$ on scale of zero to 0.25%.

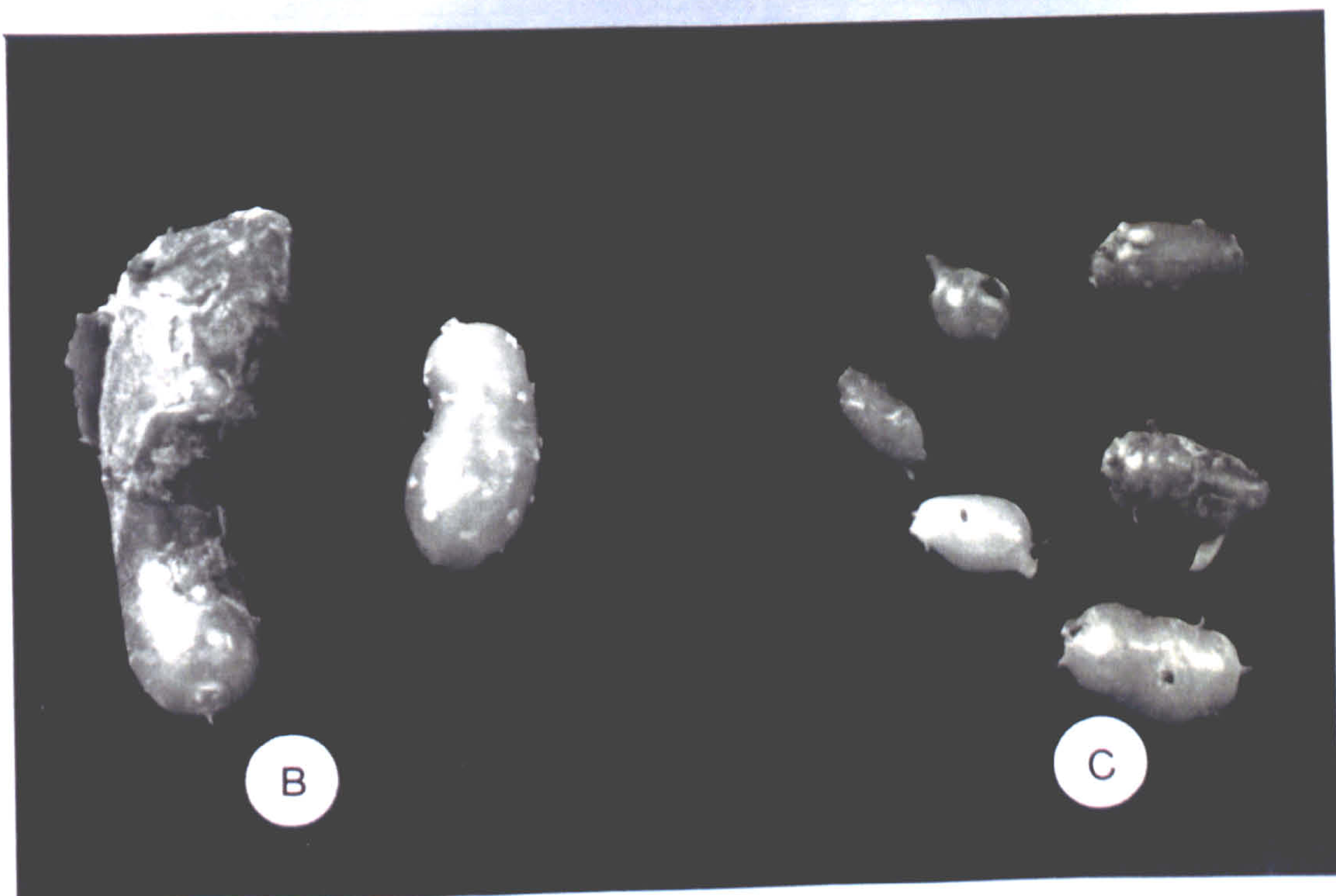
## PLATE : 8.04

above : (A) *In vitro* tuberization of potato (*Solanum tuberosum* L.) obtained from shoot cutting and cultured in MS + BAP (1.0 $mgl^{-1}$ + 8% sucrose (after 6 weeks of culture; X3).

below : *In vitro* grown microtubers of potato (*Solanum tuberosum* L.) obtained from shoot cutting cultured in MS medium supplimented with : (B) BAP 1.0 $mgl^{-1}$ + 8% sucrose; (C) BAP 1.0 $mgl^{-1}$ + 4% sucrose (after 8 weeks of culture; X2).

Plants grown in all case with diffusive ventilation.

A


B
C

When the concentration of BAP was greater than 1.5 mg $l^{-1}$ the growth of roots and shoots was inhibited, and at very high concentration (>2.5 $mgl^{-1}$) no tuberization was observed.

Tuberization was promoted by increasing sucrose concentration in the medium (Table 8.04), and the best response (4.3 per explant) was observed at a concentration of eight *per cent*. Higher sucrose concentrations (12%) delayed the onset of tuberization and slightly reduced the size and number of the tubers. Normally one tuber was produced at each node from a 'rhizome' offshoot (Plate 8.04), and often one formed also at the tip but again this was probably nodal in origin. In some plantlets the central axillary shoot first grew upright and then bent downwards. Tubers formed at the apices of some of these shoots (not shown).

**TABLE 8.04. Effects of BAP and sucrose on tuber number per replicate after 8 weeks; the culture vessels were capped with polypropylene membrane. Each value represents a mean ± SE of 5 replicates.**

| BAP ($mgl^{-1}$) | Sucrose | | |
|---|---|---|---|
| | 4% | 8% | 12% |
| 0.0 | No tuberization Well developed shoot system | No tuberization Well developed shoot system | No tuberization Well developed shoot system |
| 0.5 | 1.1±0.1 | 1.6±0.1 | 0.9±0.1 |
| 1.0 | 3.7±0.4 | 4.3±0.3 | 3.1±0.2 |
| 2.0 | 2.1±0.2 | 2.2±0.2 | 2.0±0.2 |
| 2.5 | 0.5±0.0 | 1.0±0.2 | 0.7±0.1 |

In the present investigation, a forced ventilation of natural gases in the culture vessel was applied to try to improve the tuberization technique. The results indicate that while forced ventilation did not significantly increase the number of tubers, it markedly improved the fresh weight of the tubers which was almost double when compared with that of diffusive one (Fig. 8.04). In the diffusive treatment some of the shoots became

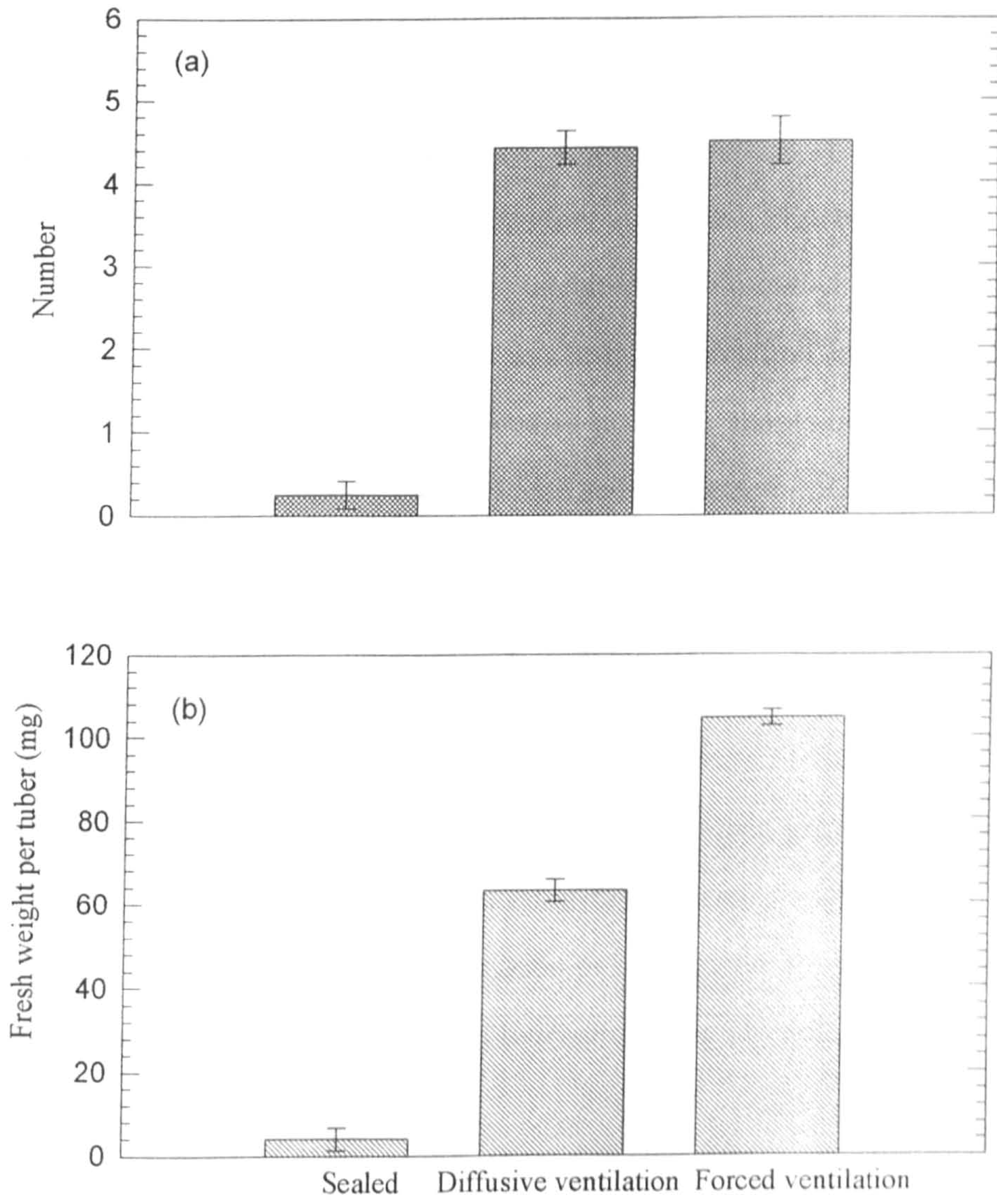



**Fig. 8.04.** Showing (a) the number and (b) the fresh weight of potato tubers grown in 60 $cm^3$ culture vessels under closed, diffusive and forced ventilation (fast flow - flow rate = 3.5 $cm^3 s^{-1}$) for 8 weeks. Each bar represents a mean ± SE of 10 - 15 replicates.

swollen in places after four weeks of culture (Plate 8.05). However, during the experiment in sealed condition no tuberization occurred.

The findings suggest that forced ventilation does improve tuberization. This may be due to increase supply of $CO_2$ during the light period and/or to the removal of accumulated ethylene from the culture vessel head-space. Although Jackson *et al* (1987) found no effect of ethylene on the induction of tuberization, in contrast, Hussey and Stacey (1984) reported that addition of the ethylene inhibitor 2-chloroethyl-trimethylammonium chloride (CCC) to the medium markedly increased tuberization in potato. They also reported that the presence of ethylene tended to make the shoots become stoloniferous (Hussey and Stacey 1981). Mingo-Castel, Smith and Kumamoto 1976 also reported that ethylene inhibits tuberization. Moreover, they showed that $CO_2$ promotes tuberization. In the present investigation no specific attempt was made to find whether ethylene had affected tuberization. However, since the numbers of tubers were not influenced by the diffusive ventilation it seems likely that the beneficial affects of forced ventilation will have been through the supply the $CO_2$ necessary for tuber growth rather than the removal of ethylene. The poor tuber initiation in sealed vessels might have been direct, i.e. due to ethylene inhibition of tuber formation or indirect, i.e. growth inhibition of the plants may have delayed their attainment of tuber-producing physiological age.

**PLATE : 8.05**

Potato stem grown-*in vitro* under diffusive ventilation (capped with a polypropylene disc) in a 60 ml culture vessel and cultured in MS + BAP (1.0 $mgl^{-1}$ + 8% sucrose; note shoots has developed a localised swelling after 4 weeks of culture (X4).

## 8.4. FINAL COMMENTS

The results have shown that the growth of potato stem cuttings can be substantially improved by means of forced ventilation of the culture vessels, and in general the best growth was achieved with the higher flow. Ethylene removal is clearly an important contributory factor in the better growth found with diffusive and forced-flow ventilation, but additionally $CO_2$ supply clearly contributes to the even better growth found with forced-flow ventilation.

Similarly tuberization was improved by forced ventilation with tuber size being the major beneficiary. Again, it is difficult to separate the contributions made by $CO_2$ enhancement on the one hand and ethylene removal on the other. However, the results have shown a positive contribution of $CO_2$ on shoot growth over and above that of ethylene removal (Table 8.02). Consequently, it seems very likely that the greater yield with forced ventilation will have owed much to the greater photosynthate production of the larger plants.

Finally it should be noted that in potato, generally, tuberization is favoured by short days and low temperature. In these experiments a 16-hour light period was provided and the temperature was 23°C. It is anticipated that the tuberization might be further improved by providing the shorter light period (e.g. 6-8h rather than 16h) and cooler temperatures: 15-18°C.

# CHAPTER - IX

## FINAL DISCUSSION

During the last decade there has been an increasing awareness that plant tissue cultures can benefit from improvements in ventilation (General Introduction). With conventional plant tissue culture capping systems the exchange of gases between tissues and the outer air can become often seriously limiting to the growth and development of callus and plantlets. This inability to grow well in culture has been said to have greatly hampered tissue culture research. Thus, establishing a method for effectively controlling the gaseous environment in the culture vessel should contribute towards high production efficiency and improve product quality, thereby considerably expanding the application of plant tissue culture techniques.

The work presented in this thesis has shown that obvious improvements in the rate of growth and quality of plant product can be produced by means of a simple method of increasing gas exchange between the culture vessel and the external atmosphere. The apparatus described and investigated here has some definite advantages over conventional systems of forced ventilation; being based upon humidity-induced diffusion it requires no complex and expensive mechanical parts e.g. pumps/cylinders, metering devices etc., little maintenance and no pipework between vessels. It requires no artificial energy supply and therefore could be useful under conditions where such a supply is difficult to obtain and thus may be particularly valuable in developing countries. Also, since the system is designed for ventilating individual vessels the risk of cross contamination is minimised.

The prototype (System I) had certain disadvantages (described in Chapter II): it was cumbersome, had a relatively slow flow rate and needed daily attention. In the new apparatus (System II) such disadvantages have been largely overcome by combining the inflow and outflow turrets in one unit, by the use of the highly water absorbent 'Oasis' material, and by a large reservoir, refilled only every 5 to 7 days. Also, in the new system

there is virtually no danger of wetting the inflow membrane and flow rates have been increased by 3 to 5-fold by using larger sized membranes and by maintaining the evaporating surface very close to the membrane. With an inflow membrane diameter of 50 mm a flow of about 5.0 $cm^3$ $min^{-1}$ can be achieved and hence the air in a 60 $cm^3$ culture vessel can be "renewed" approximately every 12 min or less. Also, both the inflow and the outflow membrane can be separated from the system before sterilization (by autoclaving) and thus it is possible to re-use the membranes for much longer periods. At present the inflow membrane assembly is fitted to the apparatus with the aid of silicone grease. An obvious improvement would include some form of screw attachment. Also, the present system is made of glass and it would be much more convenient if it could be manufactured using a light-weight material such as an autoclavable plastic.

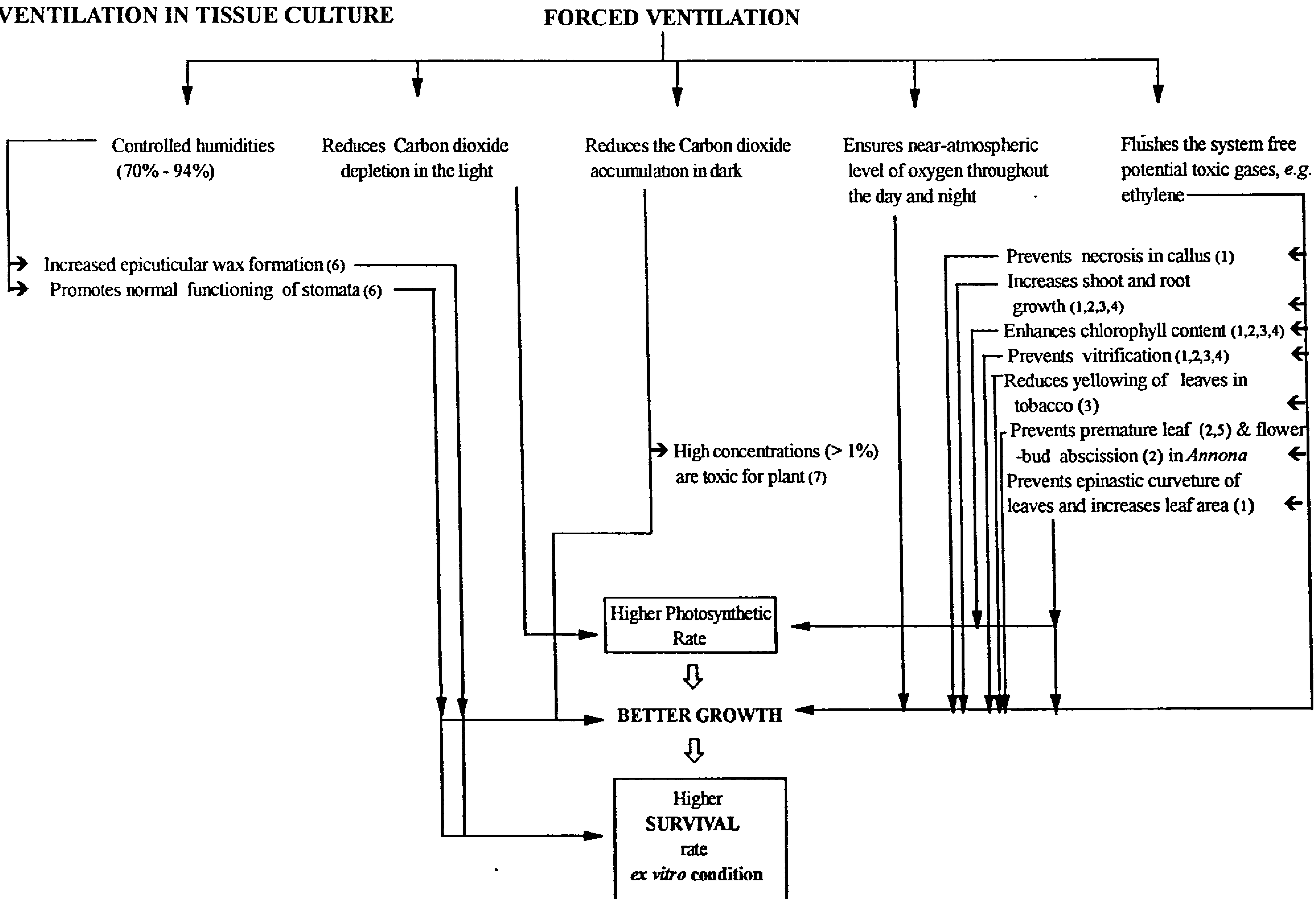
Experiments with System II have shown promising results and improvements on conventional ways of vessel capping (using either bungs or polypropylene membranes), in terms of the gaseous composition of the culture vessel atmosphere and the growth, development and physiology of cultures and plantlets as follows (see also Fig. 9.01):

(a) $CO_2$ depletion in the light can be considerably reduced, and depending on the plantlet size, $CO_2$ concentrations can be enriched and maintained up to atmospheric levels (Chapters V, VI and VII). Theoretically, in the light, $CO_2$ levels could be enriched above atmospheric by increasing concentrations *outside* the vessels, and thereby increasing photosynthesis and giving better yield. This aspect should be examined in future.

Forced ventilation also adequately reduced the accumulation of $CO_2$ in the dark, so that levels in the vessels were 350 - 650 $\mu l\ l^{-1}$ (Chapter VI).

(b) As found in cauliflower and tobacco culture (callus + plantlets) $O_2$ concentration in the sealed and diffusively ventilated vessels fell as $CO_2$ levels rose. The unavailability of oxygen has limited the growth of cultures in many species (Tate and Payne 1991; Adkins, Shiraishi and McComb 1990). Although $O_2$ was monitored only occasionally in the current experiments, on these occasions it was found that in darkness with tobacco

FIG. 9.01. BENEFICIAL IMPACTS OF FORCED VENTILATION IN TISSUE CULTURE




(1) Chapter - IV: (2) Chapter - VII; (3) Chapter - V; (4) Chapter VIII 1994; (5) Armstrong *et al* (1996); (6) Chapter VI; (7) Buddendorf-Joosten and Woltering 1994

seedlings the $O_2$ concentration was 3-4% under sealed conditions, 4-10% with diffusive ventilation and near to atmospheric level with forced ventilation (data not shown).

(c) Using $t_{50}$'s as indicators, the forced ventilation apparatus was found to flush the vessels free from ethylene, and this also would probably apply to any other potentially toxic gases. Thus the need for the use of ethylene absorbents or antagonists was eliminated (Chapter III).

(d) With forced ventilation the adverse effects of ethylene accumulation, including many aspects of vitrification, were prevented. These included depressed growth of stems, leaves and roots in cauliflower, tobacco, *Annona* and potato (Chapters IV, V, VII, VIII), leaf epinasty and the yellowing of leaves in cauliflower and tobacco (Chapters IV, V), leaf vitrification in cauliflower and potato (Chapters IV, VIII), leaf and flower-bud abscission in *Annona* (Chapter VII), failure of leaves to unfold in cauliflower and potato (Chapters IV, VIII) and poor shoot maturation in *Annona* (Chapter VII).

Also, various anatomical abnormalities were apparently prevented by applying forced ventilation. These included the production of stomata of unusually high frequency, and which were apparently permanently wide open, even in the dark (cauliflower and tobacco), comparatively small amounts of leaf epicuticular waxes in cauliflower, and short epidermal hairs in tobacco (Chapter VI).

Although the removal of ethylene was clearly an important factor contributing to the better growth found in these species when forced ventilation was applied, it was also clear that $CO_2$ enrichment in the light was also very important (Chapters IV, V)

(e) Ethylene apparently contributed also towards depressing the leaf chlorophyll levels in cauliflower, tobacco and *Annona*: Chapters IV, V, VII; the chlorophyll contents increased significantly in forced ventilation and these, together with the $CO_2$ enrichment were no doubt responsible for the higher photosynthetic rates and higher yields observed with the throughflow ventilation (Chapters IV, V).

(f) With forced ventilation, humidities in the culture tubes ranged between 70% RH and *ca.* 94%. The vitrification of cultured plantlets commonly occurring in conventional systems has also partly been correlated with too high humidities in the head-spaces, which in this study have commonly been *ca.* 100%. By changing the ratio of membrane area : head-space volume, and using different sizes of outflow membrane, controlled humidities between 50 and *ca.*100% should be attainable. These aspects should be explored further in future, particularly in connection with the "weaning" of plants and the reduction of vitrification.

In future it would be desirable to determine the water loss from the agar culture medium. It could be anticipated that with long-term culturing with diffusive ventilation, such water loss might prove to be a significant problem. For example Buddendorf-Joosten and Woltering (1996) found it necessary to control the external humidity in a new system involving a throughflow of air over vessels capped with diffusive-membranes. The forced ventilation system described in this thesis, because it can deliver humid air, might reduce water loss from the medium to an acceptable level.

Also, in any future study it would be interesting to investigate further the optimum values for $CO_2$ levels, RH and flow rate in vessels for the growth of cultures and plantlets and how these might be attained, particularly in connexion with the culturing of recalcitrant species. With this in mind it should be possible by both experiment and mathematical modelling to see how a balance could be achieved between (i) the area of inflow membrane, (ii) the area of subtending free-water surface, (iii) the depth of head-space, (iv) the area of the outflow membrane, (v) relative membrane porosities, and (vi) ambient RH and growth room temperatures and air-circulation rates.

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# APPENDIX - I

## MURASHIGE AND SKOOG (MS) MEDIUM, 1962

| CONSTITUENTS | CONCENTRATION (mg $l^{-1}$) |
|---|---|
| **Macronutrients** | |
| $KNO_3$ | 1900.00 |
| $NH_4NO_3$ | 1650.00 |
| $KH_2PO_4$ | 170.00 |
| $CaCl_2, 2H_2O$ | 440.00 |
| $MgSO_4, 7H_2O$ | 370.00 |
| **Micronutrients** | |
| $FeSO_4, 7H_2O$ | 27.80 |
| $Na_2$-EDTA | 37.30 |
| $MnSO_4, 4H_2O$ | 22.30 |
| $H_3BO_3$ | 6.20 |
| $ZnSO_4, 4H_2$ | 8.60 |
| KI | 0.83 |
| $Na_2MoO_4, 2H_2O$ | 0.25 |
| $CuSO_4, 5H_2O$ | 0.025 |
| $CoCl_2, 6H_2O$ | 0.025 |
| **Vitamins** | |
| Glycine | 2.00 |
| Nicotinic acid | 0.50 |
| Pyridoxine HCl | 0.50 |
| Thiamine HCl | 0.10 |
| M-Inositol | 100.0 |

pH adjusted to 5.8 before autoclaving.

# APPENDIX - 2

## STORAGE OF STOCK SOLUTIONS (ETHYLENE INHIBITORS)

**Silver nitrate ($AgNO_3$):** stock solution (0.1 M) was stored in dark at room temperature.

**Cobalt chloride ($CoCl_2$):** stock solution (0.1 M) was stored at room temperature.

**Silver thiosulphate (STS, $Ag_2S_2O_3$):** STS was prepared from stock solutions of 0.01 M $AgNO_3$ and 0.04 M $Na_2S_2O_3$; equal volumes of each solution were mixed to produce fresh STS for each experiment.

The pH of the all the three stock solutions were adjusted to 5.8. Stocks were filter sterilized (Millipore, 0.22 μm) and added to full strength MS medium as required after the autoclaving step.