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Components of the gaseous environment and their effects on plant growth and development *in vitro*

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1. Introduction

Several aspects of plant biotechnology have led to an increasing interest in *in vitro* culture research of plants. Micropropagation is a popular and expanding area for commercial production because it allows rapid production of genetically identical plant material and it facilitates the production of disease-free plants. In order to raise plants from genetically altered cells, *in vitro* regeneration and micropropagation systems are a prerequisite. Anther culture is an important aid for plant breeding. Furthermore, biosynthesis of secondary metabolites from plant cell cultures is an important area of research.

In order to protect the aseptic culture from infection and to prevent desiccation of the plant and the nutrient medium, *in vitro* culture is carried out in closed vessels, which unintentionally restricts the exchange of gases between the vessel atmosphere and the outside air. Growth and development of plants or explants depend not only on the composition of the nutrient medium, but may also be affected by the composition of the gaseous atmosphere [e.g. 5, 36]. However, aspects of the gaseous atmosphere have received relatively little attention. Several components, produced by either the plant material or other parts of the system, may accumulate in the vessel atmosphere. The most important factors that affect the accumulation of gases are the type and amount of plant material, the physical properties of the container and sealing, the components in the nutrient medium and aspects of the macroclimate (e.g. temperature, ventilation, light intensity).

In commercial practice many different types of vessels and sealings are used. Culture vessels are made of various materials such as glass, polypropylene, polyvinylglycine and polycarbonate with volumes ranging from 15 to 500 ml. Depending on the type of sealing, the gas exchange will be more or less restricted. Sealings can be made of several different materials, each having specific physical properties, like gas permeability and light transmittance. Sealing materials used *in vitro* are, among others, cotton plugs, cellulose stoppers, transparent film, aluminum foil, screw caps made of e.g. polypropylene or polyvinylglycine, or combinations of caps with foil or film. Due to the restriction of gas exchange by the sealing, the gaseous composition inside the vessel may significantly differ from the outside air. Gaseous components produced by the plant material, nutrient medium or container material may accumulate inside the vessel and components that are consumed or absorbed will be depleted. It has been observed that the type of vessel and particularly the type of sealing used, may have significant effects on appearance and development of the plantlets [5, 37, 62, 95]. A nutrient medium giving optimal results in one type of system (vessel and sealing) may fail to do so in another one. This indicates that the nutrient medium, the gas phase composition and the plants interact with each other to form favourable or less favourable conditions. It also suggests that manipulation or optimization of plant growth may be possible by controlling the gaseous composition in tissue culture vessels.

Several gaseous components are present in the

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vessel atmosphere. The *in vivo* atmosphere contains mainly nitrogen (78%), oxygen (21%) and carbon dioxide (0.035%). During photosynthesis plants use carbon dioxide and produce oxygen; during respiration however, plants produce carbon dioxide and use oxygen. Carbon dioxide, ethylene, and a number of other hydrocarbons have been reported to accumulate during culture [e.g. 16, 62, 79, 95]. With increasing carbon dioxide concentration oxygen depletion is observed [2, 37, 77]. However, there are also reports that show a decreased carbon dioxide concentration inside the vessels during *in vitro* culture [e.g. 23, 48].

A large number of reports on the effects of manipulation of the gaseous composition on plant quality have appeared in the last decade. High carbon dioxide levels (2%) were favourable in promoting embryo production in anther cultures from various species [39]. Other authors found positive effects on plant growth of carbon dioxide levels up to approximately 0.3% [e.g. 17, 47, 48, 50, 54]. Carbon dioxide enrichment (CDE) during *in vitro* culture is often used to promote net photosynthesis as a way to prepare the plants for transfer to soil and growth *ex vitro*. When plants are transferred, they have to adapt to *ex vitro* conditions, in particular to the lack of sucrose in the nutrient medium and reduced air humidity; this is called acclimatization. The effects of ethylene on plant growth may vary depending on the process studied. Generally, a certain amount of ethylene is required for initiation of organs, but ethylene inhibits the growth of these organs and induces senescence. An important part of the research on ethylene was done by using inhibitors of ethylene action or biosynthesis. About the presence and effects of other components which may accumulate in the vessel atmosphere relatively little is known.

Here the occurrence of different gaseous components in tissue culture vessels is discussed, with special emphasis on the effects of the different gases on growth and development of *in vitro* cultured (ex-)plants.

2. Presence and effects of different gaseous components

2.1 *Carbon dioxide*

Carbon dioxide concentrations inside the vessels alter due to respiration and photosynthesis of the plants. In the dark, CO_2 concentrations inside culture vessels increase due to respiration [e.g. 23, 37, 83]. During the light period the CO_2 concentrations may decrease depending on the photosynthetic activity of the plants.

2.1.1 *Carbon dioxide concentrations in tissue culture vessels*

In the dark, the CO_2 concentrations in vessels with different ornamental plant species increased with time and reached levels of 0.3 to 0.9% at the end of the dark period [23]. In culture vessels containing *Magnolia* plantlets a CO_2 concentration of 2 to 5% was reached at the end of the dark period [16].

For a long time it was thought that *in vitro* cultured plantlets do not have photosynthetic activity because of the ample supply of carbohydrates through the nutrient medium. However, in semi-closed vessels containing ornamental plants cultured on a sucrose-containing medium, illumination was accompanied by a significant decrease in CO_2 concentration [23]. In similar research, during the photoperiod at a photosynthetic photon flux (PPF) of 65 μmol $m^{-2}s^{-1}$, CO_2 concentrations in the vessels were about one third of the normal atmospheric CO_2 concentration [45]. Similar decreases in the CO_2 concentrations during the photoperiod were reported by other authors [17, 46, 48, 83]. Carbon dioxide concentrations may therefore drop to levels that are generally considered to be limiting to plant growth (*in vivo*). However, it is not known whether these conditions affect plant growth through photosynthesis *in vitro*. Blazková *et al.* [5] did not actually measure the CO_2 concentrations inside culture vessels but they studied growth in sealed vessels with and without addition of a CO_2 absorber (KOH). In both cases, plant growth was retarded compared to growth in vessels with a cellulose stopper; the latter allows diffusion of CO_2 into the vessel. From these results the authors argued that the retarded plant growth in tightly sealed vessels might be due to CO_2 deficiency.

Despite the decrease in CO_2 concentration that occurs during the day, an overall increase in CO_2 concentration over time is often observed. In different types of semi-closed containers with *Gerbera jamesonii* plantlets on rooting medium, CO_2 concentrations of 1.3 and 1.8% were found after four weeks; in sealed containers concentrations increased up to 13% [95]. After a culture period of 18 days of *Prunus* shoots, the CO_2 concentration in the (probably sealed) jars was over 20% [79]. In sealed flasks with shoot-forming *Pinus radiata*, the CO_2 concentration reached 20% within three weeks [53]. Jackson *et al.* [37] measured the CO_2 concentrations in culture vessels of *Ficus*

lyrata with different types of sealing. At the end of the dark period, after 23 days of culture, concentrations of approximately 0.5, 3.4 and 8.5% CO_2 were found in loose, intermediate and tightly sealed vessels, respectively. The concentrations in the same vessels at the end of the light period were 0.2, 1 and 8.5%, respectively. In all these situations the CO_2 concentrations probably are not limiting for photosynthesis. These high CO_2 concentrations (> 1%) are generally toxic to plants *in vivo*.

2.1.2 *Carbon dioxide enrichment (CDE)*

The effects of elevated CO_2 concentrations on plant growth and development *in vitro* are summarized in Table 1. This table shows the applied CO_2 concentrations, light intensities and the application method of the different treatments. If allowed by the data, a ventilation rate was calculated.

There is no standard method for CDE in *in vitro* culture. In many cases the routinely used culture vessels are placed in a chamber with elevated CO_2 concentration. Diffusion of the CO_2 into the culture vessel results in an increased CO_2 concentration in the vessel atmosphere. In most of the reports where this method is used, the concentration inside the vessel is not the same as the concentration in the chamber. Information about the concentrations in the vessels is often lacking. For example, in culture vessels with plantlets placed in a room with 0.3% CO_2, the CO_2 concentration was between 0.2 and 0.1% at midday, under a light intensity of 80–250 μmol $m^{-2}s^{-1}$. When culture vessel ventilation is not high enough, the internal CO_2 concentration decreases during the photoperiod and may still be limiting for photosynthesis when the light intensity is high. A modification of this method uses a gas diffusible film or filter. This ensures more rapid diffusion of gases while preventing contamination of the culture. Besides more rapid diffusion of the applied CO_2 into the vessel, there is another important difference between vessels with and without a gas diffusible film or filter i.e. the normal accumulation of other volatiles, such as ethylene, is much less. This may explain differences observed in the effects of CDE in different vessels. Carbon dioxide applied to routinely used vessels may act, for instance, through inhibition of ethylene action, leading to a beneficial effect on growth. Application of CDE in vessels with a film or filter may have no additional effect because no accumulation of ethylene is apparent.

Another method to increase the CO_2 concentration is by flushing gas mixtures through the *in vitro* culture system. This is called forced ventilation (Table 1). The composition of the gas mixture is exactly known and other components normally present in the culture vessel are removed. This means that, for example, ethylene and possibly other components as well, will be flushed out effectively. In case of forced ventilation, the air movement around the plants will be higher than in case of CDE through diffusion. This may have pronounced effects on net photosynthetic rate (NPR) and plant growth. The humidity of the air could also be affected when gas mixtures are flushed through the containers. The results of these three methods of CDE are difficult to compare. It is remarkable that in the cases where forced ventilation was used in CDE studies, often no effects of CDE on plant growth were observed compared to forced ventilation with 0.035% CO_2 (Table 1).

In culture tubes with asparagus and strawberry plantlets under normal culture conditions, the CO_2 concentration during the light period was well below the photosynthetic compensation point [54]. Restoration of the CO_2 level (to approximately 0.05%) through diffusion into the tubes, increased fresh weight of the plants. A similar effect of CDE on growth of these plant species was reported by Desjardins *et al.* [17] who used CDE and supplemental lighting. Growth of tobacco plantlets was promoted when CO_2 concentration was restored (and elevated up to 0.56%) at a light intensity of 40 μmol $m^{-2}s^{-1}$[84].

The concentrations of CO_2 that are used for enhancement of photosynthesis are usually below 0.3%. There are other reports, however, in which much higher CO_2 concentrations were applied. Woltering [95, 96] treated *Gerbera* and rose plantlets with up to 5% CO_2. In these experiments, plants of both genera were greener and the leaves showed less senescence when concentrations were over 1%. Micropropagation of cacao is promoted by a CO_2 concentration of 2% [21]. Also in anther and cell suspension cultures, relatively high CO_2 concentrations were found to be beneficial. Johansson and Eriksson [39] found an increased production of microspore-derived embryos when they incubated anthers of several *Anemone, Clematis* and *Papaver* species with 2% CO_2. The most favourable CO_2 concentration varies with the tested species and genera from 2 to 5% (the two highest concentrations in the tested range). Cell suspension cultures of soybean grow best with air with a CO_2 concentration between 0.4 and 5% is flushed through [31]. The effects of high CO_2 concentrations on embryo production in anthers could not be explained by changes of the pH in the

Table 1. Effects of carbon dioxide enrichment (CDE) in relation to other experimental parameters on plant growth and/or development of (ex-)plants *in vitro*

Species	CO_2 conc. (%)	Light intensity (μmol m^{-2} s^{-1})	Realization[1]	Ventillation rate[2] (h^{-1})	Effect[3]	Influenced parameters	References
Cymbidium	0.04–0.3	19–125	Diff.		+	appearance (greenness)	30
Nicotiana tabacum	0.045–0.09	70	Diff.		+	growth of all parts most for roots	66
Asparagus officinalis						growth (fresh weight)	54, 55
Fragaria × *ananassa*	0.033–0.4	80–250	Diff.		+	Co_2-fixation	
Rubus idaeus							
Asparagus officinalis						fresh weight	17
Fragaria × *ananassa*	0.06–0.3	80–250	Diff.		+	CO_2-fixation	
Rubus idaeus						stomatal density	
Cymbidium 'Reporsa'	0.1	51–230	Diff.	0.97	+	growth (dry weight) NPR[4]	50
Cymbidium 'Reporsa'	0.035–0.3	35–226	Diff.	0.97	+	NPR	51
Dianthus caryophyllus	0.1–0.15	150	Diff.	1.4	+	growth (fresh + dry weight) NPR	48
Fragaria × *ananassa*	0.035–0.2	200	Diff.	3.7	+	fresh + dry weight NPR	47
Nicotiana tabacum	0.033–2.2	40	Diff.		+	growth rate dry weight leaf/area + thickness	84
Gerbera jamesonii	0.06–5	15	Diff.	4–5	+	chlorofyll content	96
Rosa (hybrid)						inhibition senescence	
Theobroma cacao	0.08–2	45–200	Diff.		+	elongation	21
Actinidia deliciosa	0.033–0.45	30–250	CO_2 injection		+	NPR	35
Buddleia alternifolia	0–0.12	100	F.V.	9	0		13
Chrysanthemum	0.035–0.12	75–125	F.V.	9	±	dry weight	14
Rhododendron	0–0.1	39	F.V.	15,3	0		94
Saccharum officinale	0.035–0.14	40–300	F.V.	6,3	0		93
Solanum tuberosum	0.034–2	60	F.V.	10	+	growth starch/sucrose ratio	12

[1] CDE can be realized by diffusion (Diff.), injection and forced ventilation (F.V.)

[2] Ventilation rate is not always mentioned

[3] The effects of CDE on the parameters mentioned in the next column are positive (+), small but positive (±) or absent (0)

[4] NPR = net photosynthetic rate

culture medium [39]. The mechanism of the beneficial effects of high concentrations of CO_2 is unknown. It is suggested that this CO_2 might work in two ways: in promoting photosynthesis (CO_2 concentrations up to 0.15%) [e.g. 17, 54, 84] and as an inhibitor of ethylene action (concentrations above 0.15%) [22, 96].

Most studies using CDE *in vitro* have only been concerned with the effects *in vitro* but not with the effects *ex vitro*. There is a report on the effects of CDE in acclimatization on plants treated with CDE during *in vitro* culture [55]. It seems that the growth of the plantlets which were treated with high CO_2 concentrations *in vitro* is also enhanced in the acclimatization stage. Furthermore CDE usually enhances plant growth after when it is applied transfer of the plants *ex vitro* (Table 2).

In spite of the beneficial effects of CDE described above, there are also authors who could not find any positive effect of CDE on plant growth. Cuello *et al.* [13, 14] and Walker *et al.* [93] investigated the effects of a controlled environment for micropropagation of *Buddleia, Chrysanthemum* and sugarcane. From their experiments they concluded that neither CDE nor supplemental lighting enhanced growth of the plants. Also growth of *Daucus carota* and *Catharanthus roseus* cell cultures was not influenced by concentrations of CO_2 up to 2% [87].

2.1.3 *Photoautotrophic growth*

In vitro cultured plants that are provided with carbohydrates through the nutrient medium, theoretically do not need CO_2 and light for the production of dry matter. However, there are a number of reports that showed better growth of *in vitro* cultured plants under photoautotrophic conditions than under conditions with sucrose in the nutrient medium.

Growth under photoautotrophic conditions can be stimulated by CDE in combination with high light intensities. The major part of the work in this respect was published by T. Kozai and colleagues. Because of the low CO_2 concentrations observed during the light period, Kozai and Iwanami [48] concluded that *in vitro* plantlets are apparently photosynthetically active. They argued that a carbon source in the nutrient medium is only necessary when CO_2 concentration and/or light intensity are too low to sustain photoautotrophic plant growth. For optimization of photosynthesis, together with CDE the light intensity is often increased up to 200 to 300 μmol $m^{-2}s^{-1}$. The light intensity at plant level is important when the effects of CDE are investigated, but it is not always measured in the same way in the different reports. In some reports it is estimated at plant level [e.g. 84], but there are also examples of light intensity measured at vessel periphery [12], above vessels [94] or at an empty shelf in the culture room [e.g. 49]. Kozai and coworkers [49, 52] postulated that the light intensity on the empty shelf is roughly two times that of light intensity at plant level in their culture system. When light intensity is measured outside the culture vessels it is important to consider the type of container and cap materials used. Each material has a different light transmittance, therefore the light intensity and spectrum at plant level cannot easily be predicted. Another aspect of increasing light intensity is that the temperature in the culture vessels may rise. In some reports this is taken into account, but in others it is not [54]. When temperature is changed due to altered light intensities, the air movement around the plants will also be affected and possibly the diffusion of components into and out of the vessels. These changes may affect plant growth and development and it is not possible to determine which of the observed effects is caused by changes in light, temperature, air movement or CO_2 concentration. Sometimes many variations, e.g. in light intensity, sucrose concentration of the nutrient medium and CO_2 concentrations are made at the same time and treatments with more than one factor changed are compared [30, 49].

Kozai and Iwanami supplied a higher CO_2 concentration to carnation plantlets [48] by increasing the CO_2 concentration outside the vessels (0.1 to 0.15%). The resulting increase in CO_2 concentration inside the vessel combined with a high photon flux (150 μmol $m^{-2}s^{-1}$) and a low sucrose concentration (1%) in the nutrient medium promoted plant growth and NPR. Measurements of sugar concentrations in the nutrient medium (sucrose, glucose, fructose) revealed that only 2 to 8% of the initial sugar content is absorbed after 30 days of culture of carnation plantlets under a PPF of 150 μmol $m^{-2}s^{-1}$ [48]. In liquid medium with *Cymbidium* protocorm-like bodies under similar conditions, almost all the sugar in the medium was consumed within 14 days of culture [30]. During the remaining culture period, the sucrose concentration is very low and CDE would be very effective. Under low PPF and CO_2 non-enriched conditions the fresh weight of potato plantlets increased with increasing sucrose concentration. When high PPF and CDE were applied, however, the sucrose concentration in the medium did not affect plant growth [49].

Table 2. Effects of carbon dioxide enrichment (CDE) during acclimatization of *ex vitro* plants

Species	CO_2 conc. (%)	Light intensity (μmol m^{-2} s^{-1})	Effect[1]	Influenced parameters	Remarks	References
Asparagus officinalis				growth	CDE *in vitro*	55
Fragaria × *ananassa*	0.33–0.3	80–250	+	fresh + dry weight	effects in acclimatization	
Rubus ideaus						
Pelargonium						75
Spathiphyllum floribundum	0.04–0.16	50–60	+	CO_2-fixation		76
Rosa rugosa						
Asparagus officinalis	0.033–0.15	ambient	+	root + shoot dry weight	effects on dry weight only	18
		ambient + 80 SL[2]		shorter nursery period	with SL	
Fragaria × *ananassa*	0.033–0.15	ambient	+	NPR[3]		
		ambient + 150 SL		root + shoot dry weight		19
				shorter nursery period		
Vitis (hydrid)	0.035–0.12	150	+	dry weight		56
				root growth		
				leaf area		
				root/shoot ratio		

[1] The effects of CDE on the parameters in the next column are positive (+)

[2] SL = supplemental lighting

[3] NPR = net photosynthetic rate

Photosynthesis may also be affected by the carbohydrate status of the medium; photosynthetic activity of *in vitro* grown *Clematis* plants is inhibited in sucrose enriched media, compared to plants grown *in vivo* [57]. In the first period after transfer to soil, *Vitis* plants show a delayed development which is ascribed to limited photosynthesis [56]. NPRs of *in vitro* plantlets are reported to be very low in comparison with NPRs of seedlings or greenhouse plants, as e.g. for strawberry [28]. However, net photosynthesis of *in vitro* cultured plants of *Asparagus* is similar to photosynthesis in seedlings grown in a greenhouse [20]. Also *Primula malacoides* plantlets *in vitro* have a photosynthetic ability comparable to that of plants *in vivo* [82]. Our own results (unpublished) showed that photosynthetic activity of *Gerbera* plantlets is little affected by the sucrose concentration in the nutrient medium.

However, photoautotrophy does not always lead to the best plant growth compared to growth on a medium containing sucrose. Some reports show a positive influence of sucrose on plant growth despite of CO_2 enriched conditions. For example, under CO_2 enriched conditions carnation grows best when 1% sucrose is added to the medium, compared to 0 or 2% [48]. Also in tobacco plantlets growth is optimal when CO_2 enriched conditions are combined with sucrose-containing (2%) medium [84]. Kozai *et al.* [51] reported on photosynthetic characteristics of *Cymbidium* plantlets *in vitro*. They showed that CDE (0.3%) at relatively high PPFs (100 and 225 μmol $m^{-2}s^{-1}$) promotes photosynthesis and, hence, growth of chlorophyllous shoots or plants *in vitro*. These *Cymbidium* plantlets were cultured on a nutrient medium containing 2% sucrose. Better growth of *in vitro* plants under photoautotrophic conditions, than under conditions with sucrose in the nutrient medium, might be explained by the higher uptake of ions under photoautotrophic conditions, as found in strawberry plantlets [47]. However, the role of sucrose in the nutrient medium is not clear yet. Despite high CO_2 concentrations and high light intensities, sucrose may still be beneficial for plant growth *in vitro*.

To improve plant growth and survival after transfer from *in vitro* culture to soil, stimulation of photosynthesis during *in vitro* culture may be useful. Another

possibility is to stimulate photosynthesis during the acclimatization stage *ex vitro* (see Table 2). For this purpose CDE is often used in combination with supplemental lighting [19, 24, 46, 55, 56, 75]. These conditions are in most cases favourable for plant growth. Plants should be able to produce their own assimilates when sucrose is no longer available in the nutrient medium. Because of increased growth of the plantlets by CDE, the acclimatization period often can be shortened.

2.2 *Ethylene*

The effects of ethylene *in vitro* are diverse. For cell, callus and anther cultures, accumulation or addition of ethylene is often inhibitory. Addition of inhibitors of ethylene production or action is often stimulatory to growth and embryogenesis (for review see 4). Here we mainly focus on the concentrations that occur in culture vessels and the effects of ethylene on *in vitro* cultured (ex-)plants.

2.2.1 *Ethylene concentrations in tissue culture vessels*

Depending on the ethylene production of the plantlets, the ambient ethylene concentration in the air during transplanting and the type of sealing (ventilation rate) of the culture vessels, ethylene concentrations may be high or reach high values. After three weeks of culture of tomato, peach × almond hybrid, and sweet cherry, ethylene concentrations inside the vessels were between 0.4 and 0.5 $\mu l\ l^{-1}$, concentrations in vessels with grapevine were about 0.1 $\mu l\ l^{-1}$ [41]. In vessels with miniroses concentrations up to 6 $\mu l\ l^{-1}$ were found [15]. In vented tubes with *Brassica campestris* the ethylene concentration was 0.01 $\mu l\ l^{-1}$, whereas sealed tubes contained 0.30 $\mu l\ l^{-1}$ after 20 days of culture [58, 59]. The vented treatment was obtained by placing a foam plug in a 25-mm aperture in the lid; in the sealed treatment the aperture was closed with a silicone stopper. In sealed vessels with carnation, accumulation up to 0.7 $\mu l\ l^{-1}$ occurred after 4 weeks, whereas unsealed vessels contained less than 0.1 $\mu l\ l^{-1}$ [62]. In callus cultures of *Hevea brasiliensis* ethylene concentrations were over 5 $\mu l\ l^{-1}$ under confined conditions in the dark after 5 weeks [3]. In flasks with shoot-forming *Pinus radiata* cotyledons 20 $\mu l\ l^{-1}$ ethylene was found after three weeks of culture, whereas only 2 $\mu l\ l^{-1}$ accumulated in similar flasks under non shoot-forming conditions [53]. In vessels with *Magnolia* plantlets the ethylene concentration gradually increased from 0.5 $\mu l\ l^{-1}$ after one week to 2 to 3 $\mu l\ l^{-1}$ after 9 weeks [16]. In vessels with *Gerbera* plantlets on rooting medium, accumulation of 0.02 to 1.3 $\mu l\ l^{-1}$ ethylene was found, depending on the type of container and type of sealing used [95]. Ethylene concentrations inside culture vessels with *Prunus* shoots were between 5.6 and 8.4 $\mu l\ l^{-1}$ immediately after transplanting *in vitro* as a result of fumes produced by the gas flame, applied for sterilization of tools in the laminar flow cabinet [77].

2.2.2 *Ethylene production*

Ethylene is produced by cultured plant cells, tissues, organs and complete plants. Differences in the rate of ethylene production *in vitro* were attributed to the type of tissue, the physiological state of the biological material and the nature and concentration of the added growth regulators to the nutrient medium [25]. In culture flasks with *Dahlia* leaf segments and callus, grown on medium with α-naphtaleneacetic acid (NAA) and kinetin, ethylene production was proportional to the amount of tissue and was stimulated by high NAA concentrations [26]. The amount of ethylene that is produced by lavandin explants depends on the culture stage and 6-benzylaminopurine (BA) concentration in the nutrient medium [68]. The ethylene production greatly varies with species [41] and may be influenced by light [60, 68]. Tobacco callus produced about 500 and 1000 nl ethylene per gram fresh weight per 24 h in the dark and light, respectively [33]. The ethylene production by rose explants was between 3.5 and 6.5 nl per explant per 24 h [25]. The ethylene production by bromeliads is about 4.8 nl per plant per 24 h [15], the production may vary between 1.7 and 20 nl per plant per 24 h, depending on the time in culture and the concentrations of BA and indole-3-acetic acid (IAA) in the nutrient medium [91].

In addition to the plant material, other ethylene sources can be present in an *in vitro* culture system. Agar was identified as a significant source of ethylene [60, 63]. Some brands of agar released considerable amounts of ethylene when they were exposed to light [60]. The ethylene release from agar is concentration dependent: the higher the agar concentration, the higher the ethylene release [63]. Carbon dioxide cylinders used in CDE studies, may be contaminated with ethylene ranging from less than 0.1 up to 475 $\mu l\ l^{-1}$ [65]. Another possible source of ethylene is rubber after autoclaving [38], often used in caps to facilitate gassampling. Introduction of ethylene in tissue culture vessels may also come from combustion gases

produced by a gas burner used in combination with ethanol, to sterilize tools during transplanting [77].

2.2.3 *Manipulation of ethylene production, ethylene action, and accumulation in the vessels*

Ethylene accumulation in culture vessels is affected by the physical properties of the system (mainly the type of sealing) and the release by the plant material or other components in the system. Several methods are available to manipulate ethylene levels or production rates (Fig. 1).

In plants, ethylene is produced from S-adenosylmethionine (SAM) through the intermediate 1-aminocyclopropane-1-carboxylic acid (ACC). The enzyme, that converts SAM into ACC, is considered to be the rate limiting step in ethylene biosynthesis. The activity of this enzyme, ACC synthase, can effectively be blocked by treating tissues with aminoethoxyvinylglycine (AVG) or amino-oxyacetic acid (AOA), hereby manipulating ethylene production and, hence, accumulation in the vessel. Similarly, the enzyme converting ACC into ethylene, ACC oxidase, can be blocked by cobalt ions (e.g. $CoCl_2$).

Addition of ACC to the medium often results in increased rates of ethylene production. Also auxins and cytokinins may, through their direct or indirect effects on ACC synthase activity, stimulate ethylene production. Carbon dioxide, present in the container atmosphere, may stimulate ethylene production through its effect on ACC oxidase activity. Once produced, the ethylene is thought to bind to a receptor protein which mediates the response. The binding of ethylene to the receptor can be blocked by 2,5-norbornadiene (NBD) and Ag^+ ions, either applied as $AgNO_3$ or as silver thiosulphate (STS). In this way the response is blocked without a direct effect on the production. Also increased CO_2 concentrations are known to suppress ethylene sensitivity. The precise mode of action of CO_2 on ethylene sensitivity is at present unknown. The ethylene produced may diffuse out of the tissue to the gaseous atmosphere where it can be chemically removed by using, for instance, a solution of potassium permanganate ($KMnO_4$), Ethysorb (activated aluminum oxide coated with $KMnO_4$) or a mercuric perchlorate solution. These chemicals cannot be dissolved in the nutrient medium and are therefore applied by placing e.g. a vial with the chemicals in the culture vessels. Finally, ethylene may be introduced into the vessels by using ethylene gas from pressurized bottles or by adding components that generate ethylene (e.g. ethrel or ethephon, both containing 2-chlorethylphosphonic acid (CEPA) or CEPA itself) to the nutrient medium (for references see [1, 69]).

2.2.4 *Effects of ethylene on plant growth and development* in vitro

The positive and negative effects of ethylene on growth and development of *in vitro* cultured plants or explants are summarized in Tables 3 and 4. In most cases proliferation is promoted by ethylene (Table 3), but growth of the induced organs is inhibited by ethylene (Table 4). In *Brassica* species, which are generally recalcitrant *in vitro*, the effects of ethylene have been investigated by several authors. For example, Chi *et al.* [7] 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. These authors suggest that poor regeneration of cultured cells and tissues of *Brassica* may, at least partially, be attributed to ethylene produced by cultured plants. They also reported that the presence of a low amount of ethylene is required for root initiation. By using ethylene inhibitors and a number of recalcitrant *Brassica* genotypes, other authors showed also that ethylene was one of the causes of recalcitrance [8, 9, 58, 59, 73, 81].

Other negative effects of ethylene *in vitro* are the inhibition of plant growth and the enhancement of senescence. In sealed vessels with *Ficus lyrata*, ethylene accumulation was accompanied by a decrease in leaf area [37]. Also in sealed vessels with *Ficus lyrata*, ethylene was thought to increase callus formation at the expense of shoot proliferation, because the effects could be reversed by placing $KMnO_4$ in the vessel [40, 42]. Addition of STS to the culture medium of potato plantlets increased leaf size [64, 71], as well as shoot and root growth [71]. Tuberization of potato is promoted when $KMnO_4$ is added [32]. These results indicate that tuberization of potato is inhibited by ethylene. Growth of carnation explants is supposed to be inhibited by accumulated ethylene because growth enhancement occurred when $KMnO_4$ was added [62]. Shoot elongation of roses is promoted when mercuric perchlorate is present in the culture vessels, indicating that ethylene inhibits shoot growth [44]. Epinasty of a peach × almond hybrid was ascribed to accumulation of ethylene [42, 43]; adding $KMnO_4$ diminishes the epinasty. Ethylene treatment induces senescence of mini-roses [15] and causes yellowing of *Gerbera* plantlets [95]. Also in carnation, leaf yellowing is stim-

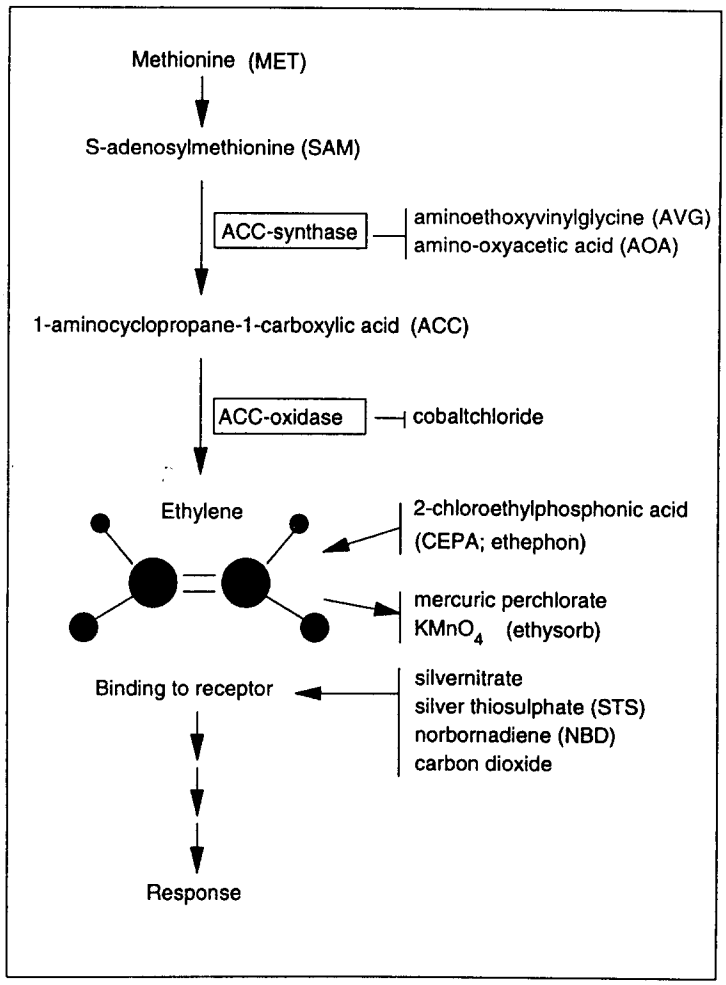

Fig. 1. Ethylene biosynthesis, action and inhibition [1, 69].

ulated when ethylene is added to the vessel atmosphere [62].

Also positive effects of ethylene *in vitro* have been described. In bromeliads ethylene suppresses the apical dominance and thereby increases the number of axillary shoots that are formed [15]. Ethylene treatment increased the bud number per bulb-scale explant of *Lilium speciosum in vitro* [90] and enhanced the development of bulb primordia at the base of regenerated shoots of tulip [85]. Shoot-forming ability of lavandin is positively correlated with the rate of ethylene production in callus tissue and micropropagation explants [68]. Gaspar *et al.* [25] observed a peak in ethylene production which coincided with the initiation of lateral shoot outgrowth from basal axillary buds in cultured rose explants. Suppression of the ethylene production by addition of AVG or $CoCl_2$ did not change this process, indicating that there is no functional relationship between axillary budding and ethylene biosynthesis. Further investigations showed that pulse treatment of ethylene enhanced proliferation depending on concentration and time of application [44]. Ethylene removal with mercuric perchlorate resulted in a reduced shoot proliferation rate, but increased shoot elongation, thereby generating a higher number of rose shoots suitable for propagation [44].

Cachita *et al.* [6] reported of several positive effects of ethylene. They investigated shoot and root formation in the presence of CEPA and concluded that ethylene promotes organogenesis (roots and shoots) and growth of carnation meristems, *Cymbidium* pro-

Table 3. Presumed positive effects of ethylene on plant growth and/or development *in vitro*

Plant species	Treatment[1]	Effect[2]	Influenced parameters	References
Dianthus caryophyllus	CEPA	+	bud + shoot formation	6
Cymbidium	CEPA	+	organogenesis	
Chrysanthemum morifolium	CEPA	+	organogenesis	
Solanum tuberosum	CEPA	+	organogenesis	
Gerbera	CEPA	+	organogenesis + growth	
Brassica	AVG	−	root elongation	7
Achmea dactilyna	AVG	−	number of shoots	15
	ethyleen	+		
Corylus avellana	methionine	0	rooting	27
	ACC	0		
	CEPA	+		
	AVG	−		
GF677	sealing	+	proliferation	43
(peach × almond hybrid)	$KMnO_4$			
Rosa	ethylene	+	proliferation	44
	AVG	0		
	ACC	0		
	$CoCl_2$	0		
Pinus radiata	sealing	+	differentiation	53
	$KMnO_4$	−	of buds	
Helianthus annuus	AVG	−	root formation	61
	STS	−		
	ACC	+		
	ethephon	+		
Tulipa	ethylene	+	bulb primordia	85
	ACC	+		

[1] For abbreviations of the treatments see text 2.2.3

[2] Effects on the parameters mentioned in the next column are stimulatory (+), inhibitory (−) or absent (0)

tocorms, *Chrysanthemum* and potato minicuttings and *Gerbera* shoots.

Ethylene was shown to be an important factor in rooting, but the experimental results have been contradictory. In some studies ethylene stimulated rooting [6, 27, 61, 70] while in other ethylene had an inhibitory or no effect on rooting [11, 42, 71, 73]. The effects of ethylene on rooting probably depend on the concentration and on the physiological stage of the explants.

2.3 *Oxygen*

There are very few reports on the influence of oxygen concentration on plant growth *in vitro*. It is expected that a moderate decrease of oxygen concentration will not effect plant growth and development. Generally with increasing CO_2 concentration in the vessels, a comparable decrease in oxygen can be expected.

In Petri dishes with rice callus the oxygen concentration was 2 to 5% after 24 days of culture [2]. In

Table 4. Presumed negative effects of ethylene on plant growth and/or development *in vitro*

Plant species	Treatment[1]	Effect[2]	Influenced parameters	References
Brassica	$AgNO_3$	+	shoot regeneration	7, 8, 9
	Ag_2SO_4	+		
	AVG	+		
	AOA	0		
	DNP	0		
	ethephon	−		
Helianthus annuus	$AgNO_3$	+	shoot regeneration	10
	$CoCl_2$	+		
	ethephon	−		
	ethylene	−		
Rosa	AVG	0	senescence	15
	ethylene	+		
Solanum tuberosum	$KMnO_4$	+	tuberization	32
Ficus lyrata	sealing	−	leaf area	37
Ficus lyrata	sealing	−	shoot proliferation	42
Peach × almond	sealing	+	epinasty	42, 43
hybride	$KMnO_4$	−	senescence	
Rosa	AVG	+	shoot elongation	44
	$CoCl_2$	+		
	ACC	−		
	ethylene	−		
Brassica campestris	NBD	+	plant development	58, 59
	sealing	−		
Dianthus caryophyllus	ethylene	−, +	growth, yellowing	
	$KMnO_4$	+, −	growth, yellowing	62
Solanum tuberosum	STS	+	leaf size	64, 71
			shoot + rootgrowth	
Cruciferae	$AgNO_3$	+	shoot regeneration	73
	AVG	+		
Gerbera jamesonii	sealing	+	yellowing	95
	ethylene	+		

[1] For abbreviations of the treatments see text 2.2.3

[2] Effects on the parameters mentioned in the next column are stimulatory (+), inhibitory (−) or absent (0)

tightly sealed vessels with *Ficus* plantlets, oxygen concentrations of approximately 10% were observed [37]. Several cultivars of *Prunus avium* (cultured at a PPF of 30 μmol $m^{-2}s^{-1}$) differed very much in the changes in O_2 concentration during *in vitro* culture. Two cultivars showed drastic O_2 consumption until the concentration reached a level of about 10% after 30 days, whereas two other cultivars showed only a slight increase in O_2 concentration up to 24% in the same period [78].

In *Torenia* stem segments adventitious buds were initiated after a short term treatment with 100% nitrogen, similar treatment with air showed no effect [86]. An oxygen tension of 5% or less stimulated plantlet production from anther cultures of tobacco [34]. In *Spiraea* the shoot number increased after treatment with 100% nitrogen (no oxygen). The stimulation of shoot proliferation by hypoxia may be due to the effects of these conditions on ethylene biosynthesis according to Norton [67]. Plantlets of *Primula* and *Chrysanthemum* cultured in 10 and 1% oxygen showed a higher NPR than those cultured in 21% [82]. Hypoxia (of 0.5 to 2% O_2) can also be applied to slow down senescence of *in vitro* (peach) plantlets during storage in order to delay the subculture and to limit the risk of genetic variation [74].

Oxygen limiting conditions resulted in suppressed growth of *Catharanthus roseus* cell cultures. However, the growth yield (g cell mass generated per g glucose consumed) was not affected by oxygen deprivation. This means that under these conditions the cells did not convert significant amounts of sucrose to fermentative products such as ethanol and this may therefore not explain the observed repressed growth [87].

An advantage of decreased oxygen concentrations is that this inhibits the growth of fungi and bacteria in the culture vessel [82]. This facilitates working under sterile conditions. However, several processes in higher plants are inhibited when oxygen concentration is low. The effects of low oxygen concentration on plant growth *in vitro* can (partly) be explained by its effect on photorespiration. For *in vivo* plants, the loss of photosynthetically fixed carbon due to photorespiration can be up to 50% in normal atmospheric O_2 concentration [98]. Photorespiration is repressed with decreasing O_2 concentration and is almost completely absent in 2% O_2 plantlets have similar photosynthetic characteristics to that of C_3 plants grown *in vivo* [20, 82]. This means that the net photosynthetic rate of C_3 plantlets *in vitro* should increase with decreasing oxygen concentration (as measured in [82]).

2.4 *Ethanol, acetaldehyde and other components*

Besides CO_2, ethylene and oxygen, other components that may influence plant growth or development can be present in the gaseous environment. There are not many reports on these "other" components, they seem to be regarded as of minor importance in normal *in vitro* culture.

When wild cherry cultures are multiplied in closed vessels, several volatile substances can be detected [79]. At the beginning of the culture period the shoots start to form ethylene and CO_2. Shoots begin to release ethanol and acetaldehyde after 6 and 14 days, respectively. After 30 days of culture of wild cherry, CO_2 reaches a concentration of 30% [80]. Formation of ethanol and acetaldehyde increased when the CO_2 concentration exceeded 20%. Accumulation of ethanol and acetaldehyde is followed by rapid tissue deterioration. At the time tissue deterioration occurred, ethane and acetone were detected inside the vessels. In control vessels (glass jars) without plants but with nutrient medium traces of ethylene, ethane, propylene, propane, methanol, acetaldehyde, ethanol and acetone were found during a three month incubation in a growth room under normal culture conditions [80].

Rice callus produces CO_2, ethylene and ethanol; one of the tested cultivars also produces small quantities of acetaldehyde and ethane [2]. Ethanol and acetaldehyde were detected in callus cultures grown in medium containing the auxin 2,4-dichlorophenoxyacetic acid (2,4-D). Independent of auxin concentration, embryogenesis in *Daucus carota* and *Phoenix dactylifera* showed an inverse relationship with the concentration of ethanol in the cultures. Exogenous ethanol reversibly inhibited embryogenesis in wild carrot callus [89]. Callus of a non embryogenic carrot strain produced much more ethanol than an embryogenic strain [88], indicating that there is a negative correlation between ethanol concentration (or ethanol production) and embryogenesis.

Volatile components that are not produced by plant material, may be present inside the culture vessels, depending on the container material used and the way tools are sterilized during transplanting. In the cases where vessels or lids are made from plastic, components, which are added to the plastic to improve weakening (plasticizers), may be released into the container atmosphere and affect plant growth. The plasticizer di-butylphthalate (DBP), for instance, was found to be very toxic to plants [29, 92]. *In vitro*, many different types of vessels are used and problems may arise

resulting in poor growth of plants as a consequence of phytotoxic components. In different container types with *Gerbera* plantlets, ethane, ethylene and propane were detected. The tested container types were made of glass, polypropylene (PP) or polyvinylchloride (PVC). In PP and PVC containers also propylene and butane were present [97]. In the PP containers an additional component, which was not present in the other vessels, was detected. This component was shown to be produced by the PP container material itself, irrespective of the presence of medium or plant material. This component was found to be phytotoxic to *Gerbera*, causing severe yellowing within two weeks of culture. The component has not yet been identified, although it is not the plasticizer DBP [97]; it is however removed by Ethysorb.

When hydrocarbons are partially oxidized or unevenly combusted and when ethanol is used for sterilization of dissecting instruments during transplanting, large quantities of biologically active components are produced [77]. This caused that culture vessels with *Prunus* shoots contained (besides ethylene and CO_2) acetaldehyde, ethanol, iso-butane, n-butane, propane and propylene immediately after transplanting.

3. Conclusions

Generally it can be stated that, in order to obtain optimal growth, the sealing of the vessels must allow sufficient ventilation to prevent significant built up of ethylene and depletion of CO_2. In addition, the container material used should be free of phytotoxic components that may diffuse into the container atmosphere.

A low concentration of ethylene *in vitro* seems to be necessary for organogenesis. Higher ethylene concentrations may have negative effects on growth and development of plants and induce senescence.

Despite the numerous reports on the beneficial effects of CDE on plant growth and development *in vitro*, the mode of action is still not clear. It is pointed out that CDE enhances NPR of plantlets *in vitro*, but theoretically there is no direct need for photosynthetic activity because of the supply of carbohydrates through the nutrient medium. Another aspect is that high CO_2 concentrations (over 1%) have a beneficial effect on plant growth and development *in vitro*, whereas these concentrations have an inhibitory effect on photosynthesis and growth of *in vivo* plants [72]. The mode of action of such high CO_2 concentrations is also not clear yet. Possibly this CO_2 may act in inhibiting ethylene action.

To really unravel the relative contribution of the different gaseous components to plant growth and development and to investigate the physiological background, the experiments should be carried out under strictly controlled conditions. In such experiments it is important that appropriate controls are part of the treatments.

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