Establishment of a photoautotrophic temporary immersion system (PA-TIS) in vitro

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Abstract

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In conventional *in vitro* cultures different problems have to be faced. Especially the rooting of woody plants is not always successful. The objective of the present work was the combination of photoautotrophic cultivation with temporary immersion, both successfully used in the past for the rooting of woody plants, to a new *in vitro* rooting system. The so-called PA-TIS, short for **P**hoto**a**utotrophic **T**emporary **I**mmersion **S**ystem, was first tested for the rooting of *Malus x domestica* 'Holsteiner Cox' and later *Kalmia latifolia* 'Ostbo Red'.

PA-TIS was provided with a closed-cycle ventilation, which was enriched with carbon dioxide. The concentration was continuously monitored and regulated not to drop below 1200 µmol mol^{-1}. Thereby, the net photosynthetic rate could be estimated, since CO_2 uptake and production were permanently recorded. The relative humidity was lowered by bottom cooling since high water availability leads to physiological abnormalities of *in vitro* shoots. The variation of the light intensity and duration were chosen as experimental parameters. Additionally, the medium composition was changed to meet the requirement of being free of exogenic carbon sources. Therefore, not only sugar- but also vitamin-free medium was used. Continuous immersion and agar cultures with or without the addition of sucrose were chosen as reference cultures.

The rooting efficiency of 'Holsteiner Cox' in PA-TIS increased significantly with higher light intensities, reaching a maximum 96 % under continuous lighting with 150 µmol m^{-2} s^{-1}. The omission of vitamins proved to have a negative effect on both plant quality and rooting, so that it was not applied in the later tests with *Kalmia*. Here, the rooting percentage was significantly higher in PA-TIS (89 %) than in the conventional agar culture containing sugar (23 %). The acclimatization success was likewise higher (85 %) than in either the sugar-free (78 %) or sugar containing (62 %) reference agar cultures.

Keywords: photoautotrophic temporary immersion system, rooting, woody plants

Kurzfassung

Etablierung eines Photoautotrophen Temporären Immersionssystems in vitro

Bei der Kultivierung von Pflanzen in vitro treten häufig Schwierigkeiten auf, wobei insbesondere bei Gehölzen die Bewurzelung von Sprossen nicht immer gelingt. Ziel der vorliegenden Arbeit war es, die in der Vergangenheit schon erfolgreich zur Bewurzelung von Gehölzen eingesetzten Ansätze der photoautotrophen Kultivierung und temporären Immersion zu einem neuartigen System zur Pflanzenbewurzelung zu kombinieren. Das sogenannte **P**hoto**a**utotrophe **T**emporäre **I**mmersions**s**ystem, kurz PA-TIS, wurde zunächst für die Bewurzelung von Apfelsprossen (*Malus x domestica* 'Holsteiner Cox') und später auch *Kalmia latifolia* 'Ostbo Red' eingesetzt.

PA-TIS wurde so konzipiert, dass die Luft in einem geschlossenen Kreislauf mit Kohlenstoffdioxid angereichert wurde. Dazu wurde die CO_2-Konzentration ständig kontrolliert und auf über 1200 µmol mol^{-1} geregelt. Somit konnte auch die Netto-Photosyntheserate über die Aufnahme und Abgabe von CO_2 kontinuierlich ermittelt werden. Die relative Luftfeuchte in PA-TIS konnte über 'Bottom Cooling', einer Kühlung des Gefäßbodens, abgesenkt werden, da hohe Luftfeuchten zu physiologischen Missbildungen bei In-vitro-Kulturen führen können. Als Versuchsparameter wurden die Änderung der Belichtungsstärke und -dauer ausgewählt. Zusätzlich wurde die Zusammensetzung des Nährmediums verändert, um dem Anspruch der Eliminierung aller externen Kohlenstoff-Quellen gerecht zu werden. Es kam so nicht nur zucker- sondern auch vitaminfreies Medium zum Einsatz. Als Referenzkulturen wurden sowohl unter kontinuierlicher Immersion als auch Agarkulturen gewählt, die mit oder ohne Saccharose als Kohlenstoffquelle versorgt wurden.

Die Bewurzelung von 'Holsteiner Cox' verbesserte sich signifikant mit Erhöhung der Belichtungsstärke, sodass in PA-TIS zuletzt unter Dauerlicht mit 150 µmol m^{-2} s^{-1} 96 % der Sprosse bewurzelten. Die Entfernung von Vitaminen aus dem Medium hatte einen negativen Einfluss auf die Pflanzenqualität, weshalb sie in der Kultur von *Kalmia* wieder zugesetzt wurden. Im Gegensatz zu der schlechten Bewurzelung in der konventionellen Kultur auf zuckerhaltigem Medium (23 %) wurden hohe Bewurzelungsraten für *Kalmia* in PA-TIS erzielt. Ebenso ließen sich Pflanzen, die aus PA-TIS kamen, zu einem höheren Prozentsatz (85 %) auf Erde etablieren als aus den Referenzkulturen (zuckerhaltig: 62 %, zuckerfrei: 78 %).

Schlagworte: Photoautotrophes temporäres Immersionssystem, Bewurzelung, Gehölze

Contents

Acronyms

(+)/(-)	agar medium with/without the addition of sugar
2,4-D	2,4-dichlorophenoxyacetic acid
A/P	active/passive ventilation
AM	arithmetic mean
BAP	6-benzylaminopurine
BGT	Biosystem- und Gartenbautechnik
BTBB	balloon type bubble bioreactor
CI	continuous immersion
DAC	digital-to-analog converter
DMC	dry matter content
Eq.	equation
FEP	fluorinated ethylene propylene
Fig.	Figure
HC	Holsteiner Cox
incl.	inclusive / included
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
IR	infra-red
KL	Kalmia latifolia
LED	light emitting diode
max.	maximum
MS	Murashige-Skoog
NAA	1-naphtaleneacetic acid
NTC	negative temperature coefficient
PA	photoautotrophic
PAR	photosynthetically active radiation, 400-700 nm
PA-TIS	photoautotrophic temporary immersion system
PM	photomixotrophic
PP	polypropylene
PTFE	polytetrafluoroethylene
RITA	récipient à immersion temporaire automatique, TIS-type
RuBisCO	ribulose-1,5-bisphosphate carboxylase oxygenase
SD	standard deviation
Tab.	Table

TDZ	thidiazuron
TIB	temporary immersion bioreactor
TIS	temporary immersion system
TRI	temporary rootzone immersion
w/o	without
WPM	woody plant medium

Physical units

ρ	density	[kg m^{-3}]
c	gas concentration	[μmol mol^{-1}, μl l^{-1}]
DIF	difference of temperature between day and night	[°C]
E	gas exchanges	[h^{-1}]
M	molar mass	[g mol^{-1}]
n	number	
$\mathbf{P}_n$	net photosynthetic rate	[μmol h^{-1} $plant^{-1}$]
PPFD	photosynthetic photon flux density	[μmol m^{-2} s^{-1}]
$\mathbf{R}_d$	respiration (dark)	[μmol h^{-1} $plant^{-1}$]
rH	relative humidity	[%]
t	time	[s]
U	voltage	[V]
V	volume	[m^3]
x	specific water content	[g kg^{-1}]

Indices

air	air
$\mathbf{CO}_2$	carbon dioxide
$\mathbf{H}_2\mathbf{O}$	water
in	inside the culture vessel
loss	loss by diffusion
m	molar
max	maximum
min	minimum
out	outside the culture vessel

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1 Introduction

In the commercial plant production, plants are proliferated and rooted in large numbers *in vitro*. In Germany, 48 million *in vitro* plants were produced in 2004 by less than 30 laboratories (Winkelmann et al., 2006). The plants are mostly treated as heterotrophic or photomixotrophic organisms and supplied with carbohydrates via the growing medium (Zobayed et al., 2004a). The addition of sugars as a carbon source leads to high infection risks since it provides a breeding ground for bacteria and fungi (Aitken-Christie et al., 1995). Therefore, plant micropropagation calls for closed vessels that permit a sterile culture. Shutting out microorganisms by keeping the vessels tightly closed results in a limited gas exchange with the cultivation room and consequently a limitation of carbon dioxide supply for photosynthesis (Desjardins, 1995).

Not only do the plants face carbon dioxide deprivation, but also an oversupply of water through the medium and evaporation of the latter. Consequently, physiological abnormalities such as hyperhydricity and diminished photosynthesis may lead to a reduced quality of micropropagated explants (Majada et al., 2000).

These problems have to be faced in order to improve plant quality. The carbon dioxide deprivation can be overcome by improving the ventilation of the vessels. The micropropagation vessels can either be kept in CO_2-enriched cultivation rooms (Heo et al., 2001) or directly ventilated (Xiao et al., 2000; Kozai and Kubota, 2001; Afreen et al., 2002a). It has been shown that ventilation improves plant quality *in vitro* by lowering the relative humidity and replenishing carbon dioxide (Majada et al., 2000). It additionally facilitates photoautotrophic cultivation, since photoautotrophy can be induced by carbon dioxide enrichment and simultaneous increase of plant lighting (Morini and Melai, 2003).

The conventional method of propagating plants in small vessels is labor-intensive and thus expensive. The enlargement of vessels for heterotrophic or photomixotrophic plant cultivation is difficult due to the aforementioned infection risk. Being less prone to contaminations, photoautotrophic cultures are ideal for scale-up. The use of porous materials as substrates has shown to be beneficial in large cultivation vessels. They were furnished with substrates such as rock wool by Kubota and Kozai (1992) and vermiculite or cellulose by Heo et al. (2001) which could be supplied with liquid medium in defined intervals. The nutrient supply is not, as in solidified media, limited and exudate control is possible. The use of liquids in plant cultivation either in submersion culture or in temporary immersion systems has been shown to improve transport from and to the plant (Ziv, 2005).

For a transfer from *in vitro* conditions to soil to succeed, the formation of roots is necessary. Roots were shown to play an essential part in high acclimatization rates. In the past, the *in vitro* rooting of woody plants has proven to be especially problematic. Kozai et al. (2005) specify the importance of the large-scale production of woody plants; they are needed for the fabrication of pulp, paper and timber. Reafforestation and thus a high number of seedlings are necessary. Woody plants are not only commercially interesting in horticulture, pomiculture, or ornamental plant production, but also for energy production. Of the 48 million plants produced in Germany, 2.6 million plants were counted among the 'woody plants' (Winkelmann et al., 2006). Hence, there is a demand for affordable and effective *in vitro* rooting protocols and systems.

Afreen et al. (2002a) introduced the temporary rootzone immersion bioreactor for the large-scale production of *Coffea arabusta* somatic embryos. The use of photoautotrophic techniques was beneficial for the plants, but the size of the reactor was unwieldy. Increasing the size of the vessels makes it difficult to autoclave them conventionally so that they have to be surface-sterilized (Zobayed et al., 1999c, 2001b). This is, again, time-consuming and inefficient in commercial production.

The present work deals with the establishment of a photoautotrophic (PA) cultivation system for the rooting of woody plants. The combination with a temporary immersion system (TIS) as a means of nutrient supply has been chosen due to the aforementioned advantages of temporary provision with liquids over solidified media. By regulating and monitoring different plant growth factors (carbon dioxide concentration, lighting, temperature, relative humidity, and immersion cycles) the microclimate within the newly developed **p**hoto**a**utotrophic **t**emporary **i**mmersion **s**ystem (PA-TIS) was regulated and optimized. The plant quality as well as biomass accumulation and rooting success were recorded and compared to photomixotrophic cultures on agar and in continuous immersion.

2 Literature Review

2.1 In vitro cultivation systems

2.1.1 The conventional in vitro culture

The production of plant material *in vitro* is commercially interesting since it poses an alternative to conventional propagation methods, especially when the plants are hard to proliferate or root. Pathogen-free material can be produced with high multiplication rates under controlled conditions, independent of seasonal influences.

Most *in vitro* plants are grown heterotrophically or photomixotrophically (Roitsch and Sinha, 2002) on solidified media, which provide the plants not only with nutrients, vitamins and growth regulators, but also carbohydrates. In a heterotrophic plant culture, an exogenic carbon source, mostly sucrose, is added to the medium. In this type of cultivation, e.g. callus or root cultures, no chlorophyll is available for photosynthesis . On the other hand, a photomixotrophic culture is also provided with an exogenic carbon source, but chlorophyllic tissue enables the plant to assimilate carbon dioxide additionally (Kubota, 2001).

The conventional tissue culture is divided into the following stages (Debergh and Maene, 1981):

- Stage 0 : Preparation of the stock plants under sterile conditions
- Stage 1 : Establishment of a sterile culture
- Stage 2 : Induction of meristems, development of buds, rapid multiplication
- Stage 3a: Elongation of buds to shoots, production of uniform plantlets
- Stage 3b: Rooting and acclimatization of the plants *ex vitro*

Plants grown *in vitro* often do not conform to the *ex vitro* phenotype. Majada et al. (2000) described the abnormalities of *Dianthus caryophyllus* after the cultivation *in vitro*: poorly developed parenchyma cells, fewer trichomes, decreased production of starch in the chloroplasts, and hyperhydricity of the shoots. Other studies also showed that the epicuticular wax and cuticle are poorly developed and the stomata deformed, resulting in a high water loss after the transfer *ex vitro*. These deformations are mostly ascribed to the conditions the plants are submitted to *in vitro*; the high relative humidity in the closed cultivation vessel and general accessibility of water from the medium are

mainly responsible for hyperhydricity and leaf malformation (Brainerd and Fuchigami, 1981; Grout and Millam, 1985; Zobayed et al., 2000a; Majada et al., 2000). The carbon source added to the medium poses a threat to the culture because it can also be a breeding ground for bacteria and fungi. It is also made responsible for irregularities in plant growth (Seon et al., 2000; Nguyen et al., 1999a).

Stage 3b, the rooting and acclimatization, often proves to be difficult, especially for woody plants. It is the most labor-intensive phase, accounting for 35 % of the production cost (Debergh and Maene, 1981). The induction of a functional root system is difficult. Roots often die when plants are transferred *ex vitro* due to the mechanical stress of medium removal (adhesion to the sensitive roots) as well as infection through microorganisms, when the medium containing sugar is not properly removed and provides for an ideal nutrient medium (Roberts and Smith, 1990; Heo and Kozai, 1999).

In order to overcome the aforementioned problems, different approaches to improve *in vitro* cultivation have been made. In the following chapters, two different methods, namely the photoautotrophic and temporary immersion culture, will be introduced.

2.1.2 The photoautotrophic in vitro culture

Photoautotrophy is a nutritional status in which organisms grow without the addition of exogenic organic compounds under assimilation of carbon dioxide via photosynthesis. Therefore, no organic compounds such as sugars or vitamins need to be added to the medium (Kubota, 2001). The advantages of cultivating plants, if possible, in a photoautotrophic culture are listed by Kozai et al. (1997): higher and steadier growth rates than in the photomixotrophic culture can be achieved since physiological and morphological problems are reduced. As the medium is sugar-free, the contamination rate is reduced and a scale-up to larger vessels is possible. The survival rate *ex vitro* rises and plant quality is thus enhanced. All these factors add up to lower production costs compared to conventional cultivation. Xiao et al. (2000) showed for *Solanum tuberosum* and *Limonium latifolium* that plants grown photoautotrophically were significantly bigger, with larger shoots, higher fresh and dry weight. In some plants, for example *Morinda lucida* and *Nicotiana tabacum*, secondary metabolites were only produced under photoautotrophic conditions as opposed to heterotrophic cultivation (Roitsch and Sinha, 2002).

Nonetheless, only a limited number of plants are grown photoautotrophically. The reasons given by Kubota (2001) are the lack of information on photoautotrophic cultivation pro-

tocols and the high costs for additional lighting, carbon dioxide enrichment, and cooling. Also, not all plant tissues are capable of photoautotrophic growth. Afreen et al. (2002b) detected a reduction of dry weight in the photoautotrophic cultivation of *Coffea arabusta* embryos during the torpedo and precotyledonary stage. Only in the cotyledonary stage and after germination, an increase of dry weight of 10 - 50 % was found. Hence it can be assumed that photosynthesis is only induced after the cotyledonary stage. Miyashita et al. (1996) state that leaves play an important role in photoautotrophic growth, because the leaf area is vital for a high photosynthetic activity.

Since many different growth factors influence the success rate and costs of the photoautotrophic culture, they will be discussed in Chapter 2.2. A general overview on which plants have already been cultivated photoautotrophically is given in Tab. A.2.

2.1.3 The temporary immersion system

The temporary immersion culture is a type of tissue culture, in which the plant tissue is only in temporary contact with the medium. Tisserat and Vandercook (1985) first described the utilization of periodic immersion. The principle of flooding is described in Fig. 2.1. A cultivation of tissue within a conventional bioreactor is difficult because of the total submersion within the medium. In solutions, the photosynthetically active tissue is deprived of air that it needs for a fully functioning metabolism since many gases such as oxygen are only slightly soluble in water (7.6 mg O_2 l^{-1} at 20 °C) (Armstrong, 1979). Oxygen deprivation is thus one of the main problems when plants are flooded for a longer time (Jackson, 2003). The solubility even decreases when the temperature or salt concentration within the medium increases. Also, a continuous immersion has a significant influence on growth and morphology of leafy explants, resulting in high hyperhydricity (Teisson and Alvard, 1999). In order to provide a homogeneous medium with an equal distribution of nutrients and gases a shaking, airlift, or stirring device has to be installed within the reactor. This results in shearing forces that may damage the tissue. By slowly pumping the medium into the cultivation vessel only a few times a day, as it is done in TIS, these forces can be avoided. Additionally, the air within the vessel is exchanged during the flooding process, resulting in a better ventilation of the explants than in most conventional propagation vessels. In Figure 2.1 this exchange process is shown. Plants already successfully cultivated in TIS are summarized in Tab. A.1.

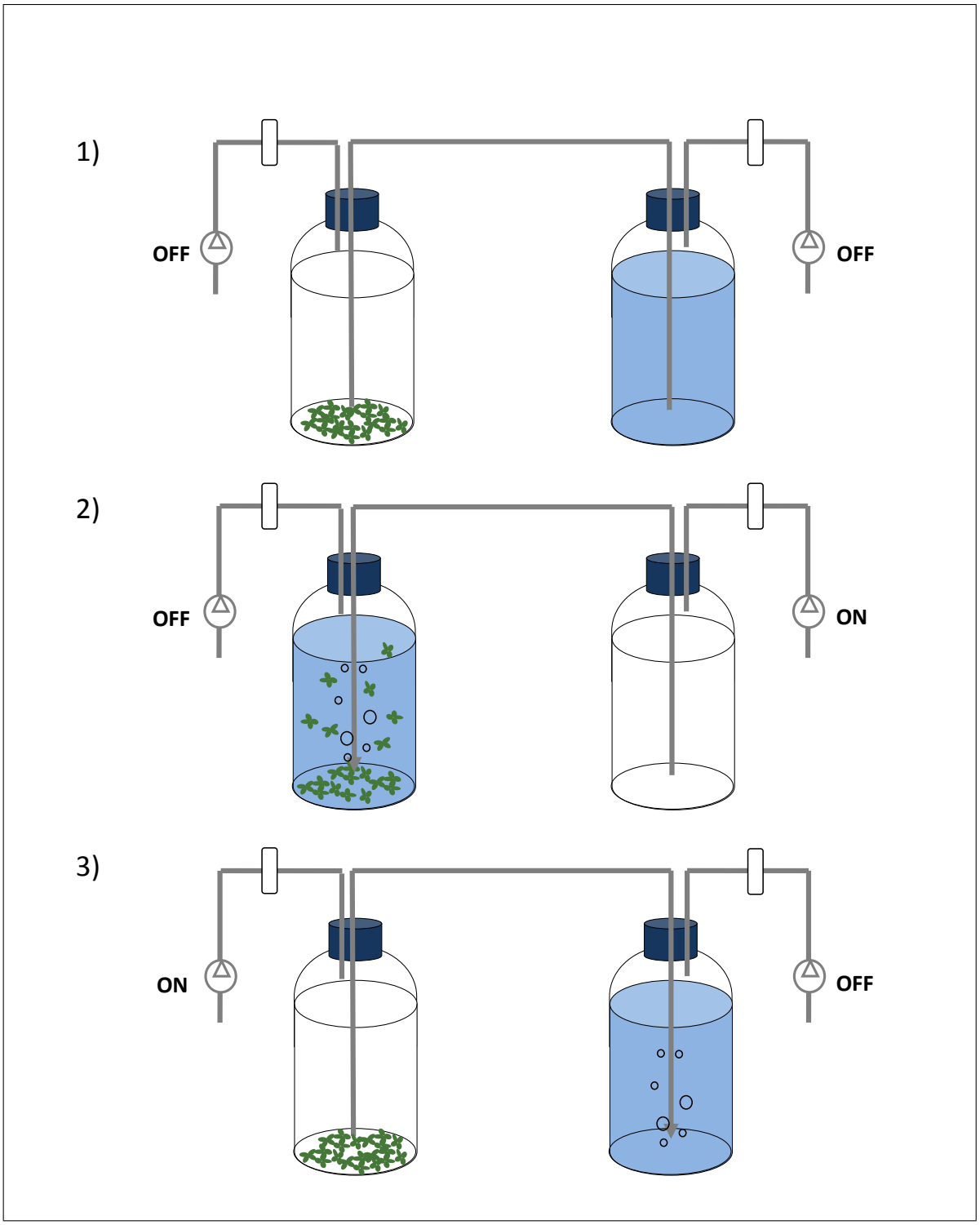


Fig. 2.1: Principle of a twin-flask temporary immersion system (TIS): 1) The cultivated tissue (left) and medium (right) are kept in separate vessels connected by a hose. The vessels are sealed off with sterile filters to prevent infections. 2) A pump is activated in order to transfer the medium from the reservoir to the cultivation vessel. Air is pumped into the bottle and displaces the medium via the hose into the left bottle. The plants are flooded. 3) The second pump is switched on when the flooding is to be reversed.

2.2 In vitro growth factors

In order to provide the ideal growth conditions for the plants to be micropropagated, several growth factors can be influenced. Even though most plants are grown on or in **media** containing sugar, **lighting** is applied at different intensities. Since photosynthesis only plays a small part in photomixotrophic cultivation and none in heterotrophic, closed vessels can be used that reduce the risk of infection, but do not eliminate it totally. **Ventilation** is thus reduced significantly, resulting in problems with the **carbon dioxide** concentration and **relative humidity** within the vessel. Depending on the culture, the **temperature** has to be adapted.

2.2.1 Carbon dioxide and ventilation

Micropropagation is usually conducted in closed vessels in order to prevent contamination, whereby the gas exchange to and from the plant is inhibited (Desjardins, 1995). This results in a limitation of carbon dioxide and accumulation of water vapor in the air. The air current speeds within these vessels are significantly slower than in greenhouses or fields, limiting photosynthesis, mass transport and transpiration (Kozai et al., 1997). Consequently, an enhancement of air movement should result in improving those factors (Kitaya et al., 2005). Huang and Chen (2005) tested many common culture vessels for their gas permeability. The results for the air exchange rates are collected in Tab. 2.1. The vessels were divided into three categories, ranging from low to high exchange rates.

Tab. 2.1: Cultivation vessel properties according to Huang and Chen (2005)

Gas permeability	Vessel type	Type of closure	Air exchange rate, h^{-1}
Low	Conical glass flask	Rubber stopper	0.015 ± 0.00020
	Conical plastic flask	Screw-cap	0.022 ± 0.00031
Intermediate	Japanese irregular box	PP-lid	0.038 ± 0.00070
	GA-7 box (tightest state)	PP-lid	0.035 ± 0.00415
	Round vessel	PP-lid	0.037 ± 0.0010
	Square box	PP-lid	0.034 ± 0.0031
High	GA-7 box (loosest state)	PP-lid	0.071 ± 0.00315
	Rectangular box	PP-lid	0.081 ± 0.005

From these results it can be concluded that vessels considered highly permeable still only provide a complete exchange of the head air space twice a day. Depending on the vessel capacity, this means that the plants are likely to experience carbon dioxide deprivation. In order to improve the air exchange rates, different approaches have been presented

throughout the literature.

2.2.1.1 Carbon dioxide

Carbon dioxide is needed as a carbon source by plants grown photoautotrophically. It is assimilated via photosynthesis and used for the production of biomass. Too much carbon dioxide results in plant damages (Fox, 1932), but up to 4500 µmol CO_2 mol^{-1} in the cultivation head space are beneficial for plant growth (Dubé and Vidaver, 1992).

To increase the carbon dioxide concentration, either a direct supply of CO_2 to the vessel or an increase of the concentration in the whole cultivation room might be considered. Nguyen and Kozai (2001b) showed for *Paulownia fortunei, Acacia mangium, Eucalyptus camaldulensis, Azadirachta indica, Garcinia mangostama L., Coffea arabusta*, and *Gmelina arborea* that elevated carbon dioxide concentrations improved plant quality and growth. The combination of high carbon dioxide concentrations (950-1000 µmol mol^{-1}) and high PPFD (230 µmol m^{-2} s^{-1}) made a photoautotrophic cultivation of *Cymbidium* possible (Kozai et al., 1987). *In vitro* carbon dioxide enrichment significantly increased the total dry weight and number of primary roots as well as the net photosynthetic rate of photoautotrophically cultivated *Eucalyptus* plantlets (Kirdmanee et al., 1995a). Regardless of the type of supporting materials used, namely agar, gelrite, plastic net, or vermiculite, the growth and survival percentage of plantlets *ex vitro* was increased.

A reproduction of the gas composition within sealed photomixotrophic culture vessels is difficult, because the carbon dioxide concentration strongly depends on the position of the vessel within the culture room (Nguyen et al., 2001). Furthermore, the diffusion rate of carbon dioxide into the vessel through natural ventilation strongly depends on the metabolic activity of the plant, the leaf area, and the vessel dimensions. Those factors are often uncontrollable; therefore, direct ventilation should be applied (Kozai and Kubota, 2001). Higher leaf areas were measured for *Coffea arabusta* under high carbon dioxide concentrations by Nguyen et al. (1999b). For different photoautotrophic orchid cultures (*Phalaenopsis, Neofinetia falcate, Cymbidium kanran, Cymbidium goeringii*), Hahn and Paek (2001) measured carbon dioxide within passively ventilated cultivation vessels every four hours by taking samples with a syringe and determining the CO_2-concentration via gas chromatography. In this case, only a monitoring, but no control of carbon dioxide took place. All orchids showed a higher fresh and dry weight accumulation than the photomixotrophic control. In order to actively influence the carbon dioxide concentration *in vitro*, Morini and Melai (2003) then again added carbon dioxide periodically via a

syringe into the cultivation vessels, and later calculated the carbon dioxide percentage of the biomass increase of *Malus pumila* Hybrid MM106 shoots. In the cultivation of peppermint and thyme, an increase of fresh weight, axillary shoots, leaf production, and rooting could be stated for 3000 μl CO_2 l^{-1} (Tisserat and Silman, 2000). The elimination of growth regulators from the medium resulted in higher quality shoots.

An important aspect of carbon dioxide uptake is the net photosynthetic rate (P_n). It rises for higher carbon dioxide concentrations, but saturation is reached at a concentration of 4500 to 5000 µmol mol^{-1} . An increase of the PPFD over 700 µmol m^{-2} s^{-1} also results in a stagnation of P_n. Low photosynthetic activity can mostly be attributed to low carbon dioxide concentrations. Therefore, the PPFD should not be increased if carbon dioxide is not equally adapted.

The net photosynthetic rate *in vitro* can be estimated using the following equation developed by Fujiwara et al. (1988):

$$P_n = K \cdot E \cdot V \cdot (c_{out} - c_{in}) \tag{2.1}$$

where K is the conversion factor of CO_2 from volume to molecular weight (e.g. 40.5 mol m^{-3} at 28 °C); E is the number of air exchanges per hour (h^{-1}) of the culture vessel; V is the air volume (ml) of the culture vessel; C_{in} and C_{out} are the carbon dioxide concentrations (µmol mol^{-1}) inside and outside the culture vessel under steady state conditions.

Zobayed et al. (1999b) on the other hand used the following equation:

$$P_n = \Delta c \cdot \frac{V}{t \cdot V_m \cdot n} \tag{2.2}$$

where Δc is the change in carbon dioxide concentration (μl CO_2 ml^{-1}) over time (t in s), V is the volume (ml) of the culture vessel, V_m is the molar volume of CO_2 at the growth room temperature, and n is the number of plants in the vessel.

It can be concluded that elevated carbon dioxide concentrations *in vitro* are beneficial for plant growth and were applied in all publications concerning photoautotrophy by actively or passively ventilating the vessels. The exact applications (active (A) or passive (P)) are summarized in Tab. A.3 (p. 178. Different application methods will be presented in the next chapter.

2.2.1.2 Increase of ventilation

By providing different methods of ventilation (humidity-induced convective ventilation, diffusive ventilation or sealed vessels), Zobayed et al. (1999b) were able to show that cauliflower seedlings grew best under the convective treatment. The shoots showed less negative symptoms and exhibited an improved net photosynthetic rate (P_n), more leaf chlorophyll, and less hyperhydricity. Plants grown in sealed vessels, on the other hand were prone to reduced leaf area, franginess, and epinasty. The photosynthetic activity, rooting, and plant quality of *in vitro* plantlets were improved with the increase of number of air exchanges and the use of florialite (a mixture of cellulose fiber and vermiculite) as supporting material (Nguyen et al., 1999a).

Majada et al. (2000) were able to push the phenotype of micropropagated carnation shoots closer to the phenotype of *ex vitro* plants by increasing ventilation. The usual variability of plants grown in sealed vessels was attributed to lacking ventilation. A control of ventilation *in vitro* resulted in an improvement of plant quality and facilitated the *ex vitro* transfer.

One way of increasing the number of air exchanges per hour is adding microporous filters to the lid, which enhance the gas supply of the vessel (Kozai and Kubota, 2001). Another one is applying direct ventilation to an *in vitro* vessel, thereby increasing the number of air exchanges significantly. In small propagation systems, aeration is sufficiently provided through hoses connected to the vessel. When the vessel is scaled up, however, air supply has to be enhanced, because the pressure drops significantly within the vessel. Zobayed et al. (2001b) introduced a system furnished with an air supply chamber (height = 2 mm) underneath the cultivation vessel including four supply hoses and air pumps. The vessel had to be surface sterilized since it was too big to be autoclaved and the material used was not thermostable.

When active ventilation is applied, a reduction of the leaf boundary layer occurs, resulting in an improvement of carbon dioxide uptake (Kitaya et al., 1996). Zobayed et al. (2002) increased the exchange rate by so-called 'forced-ventilation' from 0.1 h^{-1} to 5 h^{-1} for a 60 cm^3 vessel. This yielded a higher number of leaves, a larger leaf area and more nodes per explant as well as a reduction of leaf abscission. The latter was attributed to the lack of ethylene accumulation in the ventilated vessels. Also, the relative humidity in the vessels was reduced, which improved plant quality significantly. This was also demonstrated by Kozai and Kubota (2001) and Kozai and Nguyen (2003) for several woody plants (e.g. *Pinus radiata, Eucalyptus camaldulensis, Acacia mangium, Paulownia*

fortunei, Azadirachta indica, Coffea arabusta). In the culture of *Lycopersicon esculentum* Mill., on the other hand, forced ventilation did not lead to an improved growth rate (Kubota et al., 2001).

Zobayed et al. (2000a) found out that in a photoautotrophic culture submitted to forced ventilation, stomata were opened during photoperiods, whereas they were closed in dark periods. In contrast, plants in photomixotrophic cultures without additional ventilation lacked stomatal closure. The cuticula was also better developed in photoautotrophic plants than in the photomixotrophic ones. After transferring plants *ex vitro*, the photomixotrophic plants lost half of the stored water after 45 minutes, whereas only 19 % of the water was lost in the photoautotrophic plants within the same time. It can be concluded that the transpiration rate is much higher in leaves under photomixotrophic treatment than in photoautotrophic cultivation, especially when latter are submitted to high air exchange rates (Malda et al., 1999). In photomixotrophic plants, 82 % of the total transpiration took place through the constantly opened stomata, which resulted in a high water loss (Zobayed et al., 2000a).

2.2.2 Lighting

Explants in tissue culture have a high photosynthetic potential. In order to stimulate photosynthesis, light has to be sufficiently provided for a high photosynthetic activity (Kubota, 2001). The photosynthetically active radiation (PAR) defines the spectral range from 400 to 700 nm in which plants are supposedly most active. The photosynthetic photon flux density (PPFD), in which the intensity of cultivation lighting is measured, lies within the boundaries of the PAR and is defined to µmol m^{-2} s^{-1}. Desjardins et al. (1995) discovered that a light saturation is already achieved at a PPFD of 400 to 500 µmol m^{-2} s^{-1}. Only if a sufficient carbon dioxide concentration is paired with a high photon flux density, it can be completely exploited. Thereby, the second important factor for a successful photosynthesis is determined: a high carbon dioxide concentration within the cultivation vessel (see Chapter 2.2.1.1). Tab. A.3 covers some publications on photoautotrophic cultures and the applied PPFD.

Traditionally, fluorescent lamps are used in plant growing rooms for the lighting of cultivation vessels. The lamps in most cases are firmly installed into cultivation shelves. Depending on the culture, the PPFD is varied. In heterotrophic and photomixotrophic cultures, low PPFD between 30 to 50 µmol m^{-2} s^{-1} are applied (Fujiwara and Kozai,

1995). In order to stimulate photoautotrophic growth, an increase of PPFD to a minimum of 100 µmol m^{-2} s^{-1} was suggested by Kozai et al. (1990). High PPFD were shown to have a beneficial effect on micropropagated plants in later stages (Donnelly et al., 1985). Fuentes et al. (2005) discovered that coconut plantlets grown under photomixotrophic conditions grew best under high light intensities (halogen lamps, 400 µmol m^{-2} s^{-1}) and moderate sugar concentrations (22.5 g l^{-1}). In conventional growing rooms, the vertical distribution of the lighting is good, whereas the horizontal distribution is not, because the lamps are arranged above the cultivation vessels. Lateral lighting should therefore be applied if possible. Kozai et al. (1997) also suggested changing the lighting cycle from 16 h light and 8 h dark to 4/2 h light/dark, because short light cycles improved plant growth (Morini and Perrone, 2006). Some photoautotrophic cultures were even grown under continuous lighting (24 h) in order to accumulate chlorophyll (Roitsch and Sinha, 2002). A strong interaction of different growth factors with light has been stated by Pruski et al. (2006). They reported that the combination of sugar in the medium and light had a significant influence on the relative growth rate of potato, chokecherry and Saskatoon berry.

Lately, new approaches to lighting are feasible in commercial plant production due to the development of inexpensive light-emitting diodes (LEDs) (Nhut et al., 2003). It is possible to provide the plants with specific wavelengths that affect the plants' morphology (Heo et al., 2002a). Different color combinations (e.g. blue, red, and white) are possible and have shown to be beneficial in comparison to lighting with fluorescent lamps (Sivakumar et al., 2006).

2.2.3 Relative humidity

Not only does the lack of carbon dioxide in the vessels play an important role in plant quality, but also the high relative humidity (rH). Continuously opened stomata in photomixotrophic cultures are attributed to the high relative humidity within cultivation vessels (96 - 98 %) (Desjardins et al., 1995). Different types of ventilation can be used in order to control the relative humidity (Zobayed et al., 2005). In airtight vessels rH is almost 100 %, whereas it could be decreased to 92 % by using forced ventilation in the cultivation of *Annona squamosa* (Zobayed et al., 2002). Similar results were obtained by Zobayed et al. (1999c) for *Ipomoea batatas*, where the relative humidity in photomixotrophic and non-ventilated photoautotrophic cultures was almost 100 %, but only 88 - 95 % under force-ventilated photoautotrophic conditions. When less medium

was applied, the relative humidity even decreased to 82 - 85 %. In comparison to plants grown in closed vessels, the stomatal density of plants grown under higher air exchanges was reduced, but the stomata size was increased. High air humidity in culture vessels also resulted in shoot elongation and low humidity led to low specific leaf areas without dry weight decrease (Kozai et al., 1997). Zobayed et al. (2001a) measured the relative humidity every 7 days in tobacco and cauliflower cultures by introducing small sensors into the vessels during the dark period. Throughout the cultivation, rH increased because the plants grew and thereby the transpiration rate.

The relative humidity is strongly influenced by the cultivation temperature, because higher temperatures will lead to higher transpiration rates and evaporation within the vessels. In the following chapter, the influence of the temperature on the *in vitro* culture will be discussed. Since rH depends on the temperature, another means of decreasing the relative humidity *in vitro* can be applied and was presented by Egbers (2005). A bottom cooling device, which lowered the medium temperature to 17 °C, was installed during the cultivation of tobacco and *Phalaenopsis*, resulting in a decrease of rH from 97 - 100 % to 85 - 87 %.

2.2.4 Temperature

In cultivation rooms, the temperature is normally regulated between 20 and 25 °C. The high light intensities applied for *in vitro* cultures strongly influence the temperature within the vessels, so that it is usually 1 °C higher than outside (Zobayed et al., 2001a). Positive differences (DIF) between the day and night temperature (T_d - $T_n > 0$) were shown to have effects on the shoot height of potato plants by Kozai et al. (1997). DIF-strategies have been proven to be an effective tool to regulate plant height. For the growth of *Rehmannia glutinosa*, a DIF of 8 °C was best when high PPFD were applied during the day (Cui et al., 2000). Overall, the temperature in photoautotrophic cultures should not be too high since the net photosynthesis rate changes with the temperature (McDonald, 2003, pp.124-126). Depending on the lighting condition, the compensation point (P_n=0) for C_3-plants lies between 30 and 40 °C. C_4-plants, on the other hand, are mostly adapted to higher temperatures, so that high temperatures between 30 and 47 °C are optimal for photosynthetic activity. Therefore, a different temperature regulation should be considered when cultivating C_4-plants.

2.2.5 Growth medium and materials

Depending on the plant tissue culture, different media compositions have been defined. Many of those are commercially available as mixtures, such as Murashige and Skoog (1962) medium (MS) or Lloyd and McCown (1980) woody plant medium (WPM). Those media definitions contain inorganic components defined as macro (> 0.5 mmol l^{-1}) and micro nutrients (< 0.5 mmol l^{-1}). The macro nutrients usually are salts containing the cations of potassium (K^+), calcium (Ca^{2+}), and magnesium (Mg^{2+}), as well as the anions phosphate ($PO_4{}^{3-}$) and nitrate ($NO_3{}^-$). Nitrogen can also be added as ammonium ($NH_4{}^+$), so that ammonium nitrate is often chosen as a nitrogen source. Micro nutrients have proven to be beneficial for plant growth (e.g. iron (Fe^{2+}/Fe^{3+}), copper (Cu^{2+}), or zinc (Zn^{2+})). The availability of the provided ions depends on the pH. Therefore, the pH is adjusted within the medium according to the plants' requirements. The optimal pH-value sometimes cannot be adjusted due to problems with gelling agents that do not solidify if the pH is too low (Ebrahim and Ibrahim, 2000).

For plants grown photomixotrophically, the medium always contains an exogenic carbon source. In most cases, this source is sucrose, since it is the preferred transport form of sugars within the phloem. Plants grown photoautotrophically do not need sugars, but assimilate carbon via photosynthesis. Cui et al. (2000) showed for the cultivation of *Rehmannia glutinosa* that the elevation of the sucrose concentration from 0 or 15 g l^{-1} to 30 g l^{-1} improved the root weight, but decreased the shoot weight of the plantlets. An accumulation of sucrose in the newly developed roots was detected. Seon et al. (2000) also cultivated *Rehmannia glutinosa* and stated that a high accumulation of carbohydrates via photosynthesis or sugar uptake *in vitro* is critical for the survival *ex vitro*. After rooting plants photomixotrophically, the sugar-containing medium has to be removed completely from the plants before the *ex vitro* transfer since it would promote infection through microorganisms (Kozai and Kubota, 2001). In the sugar-free cultivation of *Solanum tuberosum* Kozai et al. (1988) were able to eliminate the loss of explants due to fungal and bacterial infections. Furthermore, no acclimatization phase was needed when transferring the plants *ex vitro*.

The effect of sucrose on plant quality is controversially discussed: Seon et al. (2000) claim that sucrose is responsible for deformation of *in vitro* plants, whereas Adelberg et al. (1999) reason that the addition of sugar does not influence the cultivated tissue negatively. In the cultivation of *Eucalyptus tereticornis* (Sha Valli Khan et al., 2002), photoautotrophic plants were compared to photomixotrophic ones. The photomixotrophic

treatment was more successful than all photoautotrophic treatments, surpassing the photoautotrophic ones in height, number of new nodia, and multiplication rate. Studies on *Coffea arabusta* showed that sugar in the medium paired with solidifying agents and low gas exchange rates inhibited photosynthesis (Nguyen et al., 1999a).

In order to initiate the different propagation stages described in Chapter 2.1.1 (p. 18), plant growth regulators are included in the medium composition. Auxins such as the naturally occurring indole-3-butyric acid (IBA) or the synthetic analogs 1-naphtaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) are added to induce cell division and/or adventitious roots. Cytokinins of either the adenine-type (6-benzylaminopurine, BAP) or phenylurea-type (thidiazuron, TDZ) enhance cell division, callus growth and differentiation (Plant Physiology Online, http://4e.plantphys.net [25.03.2010]). Most photoautotrophic cultures still depend on growth regulators, but the elimination of all carbon sources is preferable (Roitsch and Sinha, 2002).

Plants can be grown on different materials *in vitro*. The most common supporting materials are solidified media containing agar or gelrite, but inert, fibrous, or highly porous materials such as polyethylene, foam plastic, rock wool or vermiculite have also been successfully used (Kozai et al., 1997). Gelling agents such as agar or gellan gums have been considered to be growth inhibitors that induce hyperhydricity (Smith and Spomer, 1995). Even though fibrous materials were shown to allow for better growth and root development, the conventional *in vitro* culture is performed on agar media (Kozai and Kubota, 2001; Afreen et al., 2002b). Agar is mostly used for its excellent characteristics: it melts at 95 °C but only solidifies at 45 °C. This makes the handling and pouring of the agar medium quite easy.

Eucalyptus camaldulensis shoots were cultured photoautotrophically *in vitro* for 6 weeks on four different types of supporting materials (agar matrix, gelrite matrix, plastic net, or vermiculite) under carbon dioxide non-enriched or enriched conditions (Kirdmanee et al., 1995a). The growth *in vitro* was greatest in vermiculite, followed by plastic net, gelrite, and agar (in descending order) under either carbon dioxide non-enriched or enriched conditions. The growth and survival percentage of plantlets *ex vitro* were highest for plants grown on vermiculite under the carbon dioxide enriched conditions. Kozai and Kubota (2001) also stated that *Eucalyptus* shoots grew best on vermiculite followed by a simple net. A mixture of vermiculite and cellulose called florialite was introduced as a suitable porous carrier material by Afreen-Zobayed et al. (2000). The same material was used in the photoautotrophic cultivation of *Paulownia fortunei* and showed promising results compared to agar or gelrite. Porous materials generated better vascular systems

that were more adaptable to acclimatization than those formed in the less aerated gelled media (Nguyen et al., 2005).

2.2.6 Plant bioreactors

Plant tissues cannot only be cultivated on solid materials but also in bioreactors within liquid media. The nutrition transport herein is better and exudates can be controlled (Ziv, 2005). Ziv (2000) and Honda et al. (2001) gave extensive overviews on all available bioreactor types in plant biotechnology. Mechanically stirred bioreactors are seldom used in plant cultivation, because the shear forces in the medium are very high due to the stirring devices. The following stirred bioreactors have been utilized:

- aeration-agitation type (e.g. helical ribbon or centrifugal impeller bioreactor)
- rotating drum
- spin-filter

The cultivation of somatic embryos has been quite successful in so-called gas-sparged bioreactors (Ziv, 2000). Airlift bioreactors with or without tubes (draft tube, external loop) provide for a mixed and well-aired medium without the harsh shear forces of the mechanically stirred bioreactors. When leafy explants are to be propagated, a continuous contact to the liquid medium is undesirable, since it results in hyperhydricity and oxidative stress of the plant material. Therefore, non-agitated bioreactors have been designed, which provide medium discontinuously. In the mist bioreactor, for instance, medium is only sprayed on plant material. Another popular type of plant bioreactor, the temporary immersion system, and its functionality has already been introduced in Chapter 2.1.3.

In temporary immersion systems not only somatic embryos (Cabasson et al., 1997; Etienne et al., 1997; Etienne-Barry et al., 1999), but also shoots have been successfully cultivated (Lorenzo et al., 2001; Escalona et al., 2003; González-Olmedo et al., 2005; Roels et al., 2005). The plant quality was strongly influenced by the contact time with the medium. Damiano et al. (2002, 2003) showed that the best results for different fruit types were attained with immersions of 60 minutes per day in a twin-flask system. The cultivated fruits were strawberry, apple, pear, peach, cherry, plum, raspberry, and papaya. For strawberries, the shoot number in TIS could be doubled in comparison to agar cultures. *Ananas comosus* L. Merr was immersed by Escalona et al. (1999) every three hours for two minutes in a twin-flask system with 200 ml of medium per explant. The multiplication rate and fresh weight under these conditions was much higher than

under continuous immersion. When Lorenzo et al. (1998) changed the propagation type from continuous to temporary immersion (every 9 h for 2 min), a significantly higher number of sugarcane shoots was produced. The highest multiplication rate in TIS (23.9) was found for 50 ml culture medium per explant, whereas only a rate of one to four was achieved in the liquid culture.

2.3 Model plants for the establishment of PA-TIS

2.3.1 Malus x domestica

The first plant to be cultivated in the present work was the apple *Malus* × *domestica* Borkh. cv. 'Holsteiner Cox'. Apples belong to the family of *Rosaceae* and the subfamily *Maloideae*. Most commercially significant apples belong to the aforementioned *Malus* × *domestica* Borkh. (Way et al., 1990). Many approaches have been made to propagate and root apple rootstock and scion cultivars *in vitro* since the vegetative propagation of rootstocks via layering is inefficient (Pasqual et al., 2000). *In vitro* rooted *Malus* shoots have shown to perform better than *ex vitro* rooted microcuttings concerning growth as well as survival (De Klerk, 2000).

The cultivation of *Malus* is mostly conducted in closed vessels containing agar medium. Sugars, e. g. sucrose, are added to the medium as carbon source. The effect of different sucrose concentrations (0, 15, 30, 45, 60 g l^{-1}) on *Malus* × *domestica* Borkh. cv. 'Gala' was determined by de Medeiros Rodrigues et al. (2006). The highest multiplication rate was found for a concentration of 30 g l^{-1}. Without the addition of sugar, the explants died. Sotiropoulos et al. (2006) tested M9 and MM106 rootstocks on their reaction to sucrose or sorbitol in the medium. For both rootstocks, the multiplication rate, shoot length, and fresh weight was significantly higher on media containing sorbitol than on media with sucrose. The MM106 rootstock was also used in the study by Molassiotis et al. (2006), in which the effects of different osmotic components in the medium (NaCl, KCl, mannitol, sorbitol), added corresponding to an osmotic potential of -1.0 MPa, were examined. All components had a decreasing influence on the water content of the plantlets (water content of control: 94.4 % and water content with NaCl-medium: 88.3 %).

The induction of rooting *in vitro* is mostly achieved by adding auxins to the medium, particularly indole-3-butyric acid (IBA). Additionally to describing the effects of IBA on rooting of the cultivar 'Jonagold', Druart et al. (1982) showed that a cultivation in continuous

darkness induced higher percentages of rooted plantlets than under a 16/8 h light/dark cycle (80.3 % as opposed to 39.2 %). The maximum number of roots per 'Jonagold' shoot was 4.4. The effect of IBA-containing media, in which agar was either substituted for guar or cassia galactomannan, was described for the rootstock 'Marubakaido' by Lucyszyn et al. (2006). Significantly higher root numbers were achieved when IBA was added to the medium, but an increase of the IBA-concentration led to shorter roots. Ethylene perception inhibitors ($AgNO_3$, aminoethoxyvinylglycine and $CoCl_2$) were also shown to enhance *in vitro* root formation of apple shoot cultures (Ma et al., 1998).

The apple rootstock MM106 (apple Northern Spy × Paradise M1) was cultivated under a 4/2 h light-dark cycle. More and longer roots were produced under these conditions than under 16/8 h light-dark (Morini and Perrone, 2006). Comparing agar, sand, and vermiculite as carrier material, Pasqual et al. (2000) showed that M7 rooted best on agar with sucrose concentrations of 3 or 6 g l^{-1}. Similarly, M9 was tested on different materials: agar, vermiculite, and vegetary ash. In this case, the porous material was shown to have a beneficial effect on rooting (88 % instead of 79 %). On agar, the roots were longer, but on vermiculite, more secondary roots had developed (Vieira et al., 2007). A protocol for rooting *Malus* × *domestica* Borkh. cv. 'Holsteiner Cox' was established by Szankowski (2002). IBA-concentrations of 3 - 9 µmol per liter MS agar medium resulted in 100 % rooting efficiency. The rooting medium described in this work was used in the present study.

Some approaches have been made to cultivate *Malus* shoots under photoautotrophic conditions or in temporary immersion systems. They will be presented in the following chapters.

2.3.1.1 Photoautotrophic cultivation

Malus pumila MM106 shoots were propagated under short-day conditions (8h day/ 16h night), but under high PPF (210 µmol m^{-2} s^{-1}) and CO_2 (1000-11000 µmol mol^{-1}) by Morini and Melai (2003). The sugar-concentration in the medium was varied; either 0 or 30 g l^{-1} were added. Carbon dioxide was introduced periodically via a syringe into the cultivation vessels and later the CO_2-percentage of the biomass gain was calculated. The total fresh weight increase was lower in the photoautotrophic treatments than under photomixotrophic treatments independent of the carbon dioxide enriched and non-enriched conditions. The addition of carbon dioxide to the photomixotrophic culture proved to be beneficial and increase the accumulation of biomass.

2.3.1.2 Cultivation in temporary immersion systems

Shoots of the rootstock M9 'EMLA' were either continuously immersed (with net) or temporarily immersed in an ebb and flood system in a so-called bubble-type balloon bioreactor (BTBB). Both treatments were conducted with and without ventilation. The best multiplication rate and growth were found under continuous immersion, but also a high hyperhydricity of the shoots. The hyperhydricity could be reduced by applying temporary immersion and furthermore by ventilation. After the multiplication, the shoots were rooted in a hydroponic culture for 30 days. More than 90 % of the plantlets developed roots and could be acclimatized. The temporary immersion resulted in lower hyperhydricity of the explants compared to the continuous treatment (Chakrabarty et al., 2003, 2007).

Not only rootstocks, but also *Malus* scion cultivars were multiplicated in TIS. The multiplication rate of 'Gala' was much higher in TIS (7.6 or 10.0 per original explant, depending on the flooding time) than on agar (5.3) or in continuous immersion culture (4.3) (Damiano et al., 2002). For the rootstock 'Jork 9', the opposite trend was stated, with a higher multiplication rate on agar (10 per original explant) than for both TIS-treatments (2 or 8) or a continuous immersion culture (3) (Damiano et al., 2003). *In vitro* shoots of the rootstock M26 were also propagated in TIS; the commercially available RITA®-vessels were utilized. For higher BAP and IBA-concentrations the multiplication rate increased, but the shoot length decreased. The growth regulators played an important role in the multiplication. Rooting was initialized by incubating the shoots for four days in darkness in IBA-containing medium (1.2 µmol l^{-1}), and later transferring them to light without IBA. The rooting succeeded for 91 - 100 % of the plants, depending on the pretreatment (Zhu et al., 2002, 2005).

It can be concluded that *Malus* shoots can be cultivated both photoautotrophically and under temporary immersion. Since the addition of carbon dioxide proved to be beneficial in a conventional agar culture (Morini and Melai, 2003) and high rooting efficiencies were obtained in TIS (Zhu et al., 2002, 2005), *Malus* should be a good model plant for testing the effects of the newly developed system presented in this study.

2.3.2 Kalmia latifolia

The second plant to be tested in the PA-TIS was *Kalmia latifolia* cv. 'Ostbo Red'. *Kalmia* belongs to the *Ericaceae* family and is an evergreen understory shrub found in the southern Appalachian Mountains (Eppard et al., 2005). The rooting of *Kalmia* shoots *in vitro*, especially of the cultivar 'Ostbo Red' is difficult (Pavingerová and Šedivá, 1999). Williams and Bilderback (1980) tested the rooting of *Kalmia* stem cuttings *ex vitro* in different months under the influence of different growth regulators. The application of 0.1 % 2,4,5-trichlorophenoxypropionic acid (fenoprop) in September resulted in the highest rooting efficiency (55 %), followed by plants treated with a liquid IBA-dip (1 %) in the same month. The month, in which the rooting was conducted, had a strong influence, with much lower root yields down to 12 % in December. Since micropropagation *in vitro* allows for cultivation independent of seasons, a better rooting should be expected. Lloyd and McCown (1980) developed the woody plant medium (WPM) especially for the micropropagation of *Kalmia*, providing the plants with less salts than the Murashige and Skoog (1962) medium, for instance. Pavingerová and Šedivá (1999) used WPM for the micropropagation of *Kalmia latifolia* cv. 'Ostbo Red' including 20 g l^{-1} sucrose, 40 mg l^{-1} adenin sulfate and 5 mg l^{-1} 2iP. The plants were grown under 90 µmol m^{-2} s^{-1} and 22 °C. Stem segments were later successfully transformed with *Agrobacterium*. The rooting of *Kalmia latifolia in vitro* was described by Riechers (1993). The addition of 1 ppm IBA or IAA to WPM-medium resulted in up to 70 % rooting as compared to under 10 % success without any growth regulators. In previous studies at the Tree Nursery Science Section (Leibniz Universität Hannover), *Kalmia latifolia* cv. 'Ostbo Red' rooted even less efficiently (25 %).

To date, no studies on the photoautotrophic propagation or cultivation under temporary immersion of *Kalmia* have been published, so that the establishment of a successful rooting protocol for *Kalmia* in PA-TIS, combining both photoautotrophy and temporary immersion, would be innovative.

3 Objectives

The aim of the present study was the establishment of a functional photoautotrophic cultivation system for the rooting of woody plants. In previous studies at the BGT (FG Biosystem- und Gartenbautechik, Leibniz Universität Hannover), the use of a passive ventilation system has been suggested for the photoautotrophic cultivation of tobacco and *Phalaenopsis* (Egbers, 2005). Yet, the low growth rates of the photoautotrophic cultures compared to the photomixotrophic ones showed that this kind of ventilation was insufficient for a successful photoautotrophic cultivation of either tobacco or *Phalaenopsis*. Promising results have been presented in the past for the application of active or forced ventilation to photoautotrophic cultivation vessels with CO_2-enriched air by Fujiwara et al. (1988), Kirdmanee et al. (1995b), Zobayed et al. (1999c), Nguyen et al. (2000), Zobayed et al. (2000a), Zobayed et al. (2000b), Heo et al. (2001), Nguyen et al. (2001), as well as Xiao and Kozai (2004). Afreen et al. (2002a) have shown that a photoautotrophic cultivation of *Coffea arabusta* somatic embryos was possible in the so-called temporary rootzone immersion (TRI) bioreactor, in which the embryos were cultivated on florialite (a mixture of vermiculite and paper pulp suggested by Afreen-Zobayed et al. (2000)). Therein, the material was wetted every six hours for 15 minutes. The combination of a photoautotrophic cultivation with a temporary immersion system without a supporting material thus seemed feasible.

The PA-TIS was designed to overcome problems that had been detected in other systems. The main requirement for the cultivation vessel was facile handling, since working in a sterile environment calls for vessels that can be easily opened and filled. The floating of plant material during the flooding with medium had to be eliminated, because turbulences leads to unwanted clustering of the plants, which may lead to underprovision of the material. The plants entangle, especially when rooting is induced, resulting in problems of later separating them. Therefore, a carrier system had to be designed, which would be versatile enough to allow for a cultivation of high numbers of small or large explants possible without complicating the handling. The ventilation of the vessel was designed in a closed loop, in which the air was continuously controlled and provided with carbon dioxide whenever necessary. Thereby, a loss of cultivation medium through evaporation should be reduced and the use of carbon dioxide limited. For the continuous monitoring and control of different plant growth factors such as carbon dioxide, relative humidity (rH), temperature and immersion time had to be implemented. A scale-up had to be achieved with the same or even enhanced control system, in order to produce more plants.

4 Materials and Methods

In this chapter, the model plants used for testing the newly developed photoautotrophic temporary immersion system (PA-TIS) will first be introduced with the respective cultivation conditions. Afterwards, the establishment of the PA-TIS will be described in detail.

All equipment and consumables used in the experiments are shown in Tab. A.1.1 and A.1.2 (p. 174). All chemicals used for the preparation of the different media are listed with the according producers or distributors in Tab. A.1.3 (p. 175).

4.1 Plant material

4.1.1 Malus x domestica Borkh. cv. 'Holsteiner Cox'

Apple shoots (*Malus* x *domestica* Borkh. cv. 'Holsteiner Cox') were chosen in this study as model plants, because *Malus* shoots had already been successfully propagated photoautotrophically (Morini and Melai, 2003) and in temporary immersion systems (Damiano et al., 2002, 2003). Therefore, they were ideal for the preliminary testing and establishment of the system. Prof. Dr. Iris Szankowski and Karin-Ingrid Lein, Fruit Science Section, Leibniz Universität Hannover, kindly provided the original 'Holsteiner Cox' shoots.

4.1.2 Kalmia latifolia cv. 'Ostbo Red'

After the successful establishment of PA-TIS for the rooting of *Malus*, the system was tested on plants that traditionally do not root well *in vitro*. In previous tests, the rooting success of *Kalmia latifolia* cv. 'Ostbo Red' shoots did not surpass 25 % (Winkelmann, personal communication, 11.06.2009). Since neither a photoautotrophic nor temporary immersion culture protocol for *Kalmia* has been published to date, the results of the present study are an innovative research in this area. *Kalmia* shoots were kindly provided by Prof. Dr. Traud Winkelmann and Friederike Schröder, Tree Nursery Science Section, Leibniz Universität Hannover.

4.2 Media and cultivation conditions

All media used for the different developmental stages of either 'Holsteiner Cox' or *Kalmia latifolia* were prepared with deionized water. They were sterilized for 20 min at 121 °C (V-100, Systec).

4.2.1 Multiplication stage

For 'Holsteiner Cox', the multiplication medium according to Szankowski (2002) was chosen with the omission of additional myo-inositol (0.5 g l^{-1}) (hence referred to as modified) (Tab. 4.1). The pH was adjusted to 5.6 - 5.8 before the addition of plant agar, which was added separately to each bottle of medium (1 l per bottle). For the proliferation of plant material, 5 shoots were cultivated on approximately 75 ml of modified multiplication medium (Tab. 4.1) per vessel (500 ml plastic cup, Huhtamaki) for 4 to 5 weeks. The multiplication rate was about 2 to 3 shoots per original explant.

Tab. 4.1: Multiplication medium for *Malus* and *Kalmia* shoots

Multiplication medium *Malus x domestica*, modified		
Murashige and Skoog salts incl. vitamins	[g/l]	4.4
BAP	[mg/l]	1
IBA	[mg/l]	0.1
Sucrose	[g/l]	30
Plant agar	[g/l]	8
pH(with 1 N KOH)		5.6 - 5.8
Multiplication medium *Kalmia latifolia*		
Lloyd and McCown WPM salts	[g/l]	2.36
Lloyd and McCown vitamins	[mg/l]	10.4
BAP	[mg/l]	1.8
IBA	[mg/l]	0.2
Myo-Inositol	[g/l]	0.05
Sucrose	[g/l]	20
Plant agar	[g/l]	6
pH (with 1 N KOH) after the addition of agar		5.0

The multiplication medium for *Kalmia latifolia* was prepared according to Winkelmann (personal communication, 30.06.2009). The pH was adjusted to 5.0 **after** the addition of plant agar in each bottle of medium (1 l per bottle). In the proliferation stage, 20 plants were grown on 50 ml of multiplication medium (Tab. 4.1) in twist-off jars for at least six weeks.

4.2.2 Rooting stage

Tab. 4.2: Rooting medium for 'Holsteiner Cox'

Rooting medium		**MS agar (+)**		**MS agar (-)**		**PA-TIS**	
Vitamins		incl.	no	incl.	no	incl.	no
Murashige and Skoog salts	[g/l]	-	4.2	-	4.2		4.2
incl. vitamins	[g/l]	4.4	-	4.4	-	4.4	
IBA	[mg/l]	0.3	0.3	0.3	0.3	0.3	0.3
Sucrose	[g/l]	30	30	-	-	-	-
Plant agar	[g/l]	8	8	8	8	-	-
pH (with 1 N KOH)				5.6 - 5.8			

After the successful multiplication, the 'Holsteiner Cox' shoots were cut to 4 - 6 leaves per explant and transferred into the different cultivation vessels containing the different rooting media declared in Tab. 4.2. On average, the shoots weighed 57 ± 18 mg. All conditions, under which the shoots were tested, are summarized in Tab. 4.3. In the PA-TIS, the shoots were periodically supplied with 2.5 l of sugar-free rooting medium (see Chapter 4.4.5). As reference, shoots were grown conventionally in polystyrene cups (Crystal Clear, 500 ml, Huhtamaki) on agar rooting medium with and without the addition of sugar and vitamins (Tab. 4.2) as well as in suspension on a shaker (50 rpm).

Tab. 4.3: Cultivation conditions for 'Holsteiner Cox' shoots

Treatment	**Medium**	**Agar**	**Sugar**	**Vitamins**	**Number of shoots**	**PPFD** ($\mu mol\ m^{-2}\ s^{-1}$)	**Replications**
MS susp. (+)	MS	-	+	+	10x4	75	3
MS susp. (-)	MS	-	-	+	10x4	75	3
MS agar (+)	MS	+	+	+	5x8	75	6
MS agar (-)	MS	+	-	+	5x8	75	6
PA-TIS	MS	-	-	+	55	115 + 75	3
MS agar (+)	MS	+	+	+	5x8	75	3
MS agar (-)	MS	+	-	+	5x8	75	3
PA-TIS	MS	-	-	+	55	115 + 100	3
MS agar (+)	MS	+	+	-	5x8	75	3
MS agar (-)	MS	+	-	-	5x8	75	3
PA-TIS	MS	-	-	-	55	150	7
MS agar (+)	MS	+	+	-	5x8	75 (cont.)	3
MS agar (-)	MS	+	-	-	5x8	75 (cont.)	3
PA-TIS	MS	-	-	-	55	150 (cont.)	8

In all cases, the 'Holsteiner Cox' plantlets were cultivated for four weeks. Subsequently, the plants grown on agar medium were cleaned with deionized water, wiped with a paper towel, photographed, and measured. The maximal leaf length, root length, and number

of roots were recorded. The fresh weight was determined with a precision scale (Kern). For the determination of the dry weight, the plants were dried in a drying oven for 48 hours at 80 °C. Every single plant was weighed again after cooling.

The conditions, under which the plants were grown, will be introduced in Chapters 4.4.3 to 4.4.8.

Tab. 4.4: Rooting medium for *Kalmia latifolia*

Rooting medium		WPM agar (+)	WPM agar (-)	PA-TIS
Lloyd and McCown WPM salts	[g/l]	2.36	2.36	2.36
Murashige and Skoog vitamins	[mg/l]	10.4	10.4	10.4
IBA	[mg/l]	2.6	2.6	2.6
Sucrose	[g/l]	20	-	-
Plant agar	[g/l]	6	6	-
pH (with 1 N KOH) after the addition of agar				5.0

Later, the shoots were cut to 2 - 3 nodes per explant (± 5 mm) and transferred into the respective vessels (Tab. 4.5) for the rooting.

Tab. 4.5: Cultivation conditions for *Kalmia* shoots

Treatment	**Medium**	**Agar**	**Sugar**	**Vitamins**	**Number of shoots**	**PPF** (µmol m^{-2} s^{-1})
WPM agar (+)	WPM	+	+	+	3x20	75
WPM agar (-)	WPM	+	-	+	3x20	75
PA-TIS	WPM	-	-	+	80	150

The shoots were periodically supplied with 2.5 l of sugar-free rooting medium in the PA-TIS vessel (see Chapter 4.4.5). Other *Kalmia* shoots were grown conventionally on agar rooting medium with and without the addition of sugar (Tab. 4.4).

For all three treatments, the duration of cultivation was fixed to six weeks. Afterwards, the plants were cleaned with deionized water, wiped with a paper towel, photographed and measured. The shoot length, root length, and rooting intensity (strong, weak, none) were recorded. The fresh weight was determined with a precision scale (Tab. A.1.1, p. 174). All plants were transferred *ex vitro*; therefore no dry weight was quantified. They were grown on peat substrate for four weeks, whereupon the acclimatization rate was recorded.

4.3 Stomata analysis

In the second setup (4.5), 'Holsteiner Cox' plantlets were either adapted to darkness (dark period) or light (photoperiod) after four weeks of cultivation in PA-TIS under a PPFD of 150 µmol m^{-2} s^{-1} or in the reference vessels MS agar (+)/(-) under 75 µmol m^{-2} s^{-1}. Newly formed leaves (third and fourth leaf from the apex) were cut off the adapted plantlets and analyzed via leaf impression. Since the imprint-method is destructive, the same leaf could not be tested under both conditions, but leaves from the same plant were analyzed instead. Therefore, a drop of super glue was dispensed on a glass slide. The leaves had to be dried superficially in order to provide a good contact with the adhesive. They were pressed with the underside into the glue for 20 seconds and then carefully removed. The imprints were analyzed under a microscope (Primo Star LED) under 40x magnification and pictures were taken. The pictures were examined with GIMP (GNU Image Manipulation Program 2.6.5) by means of area detection. The leaf area as well as the stomatal size and opening were recorded, and the different treatments were compared to each other. The criteria for classifying the stomatal opening are given in Fig. 5.21 (p. 93).

4.4 Experimental setup and realization

4.4.1 The photoautotrophic temporary immersion system (PA-TIS)

The photoautotrophic temporary immersion system developed in the present work is based on the principle of a twin-flask system (Fig. 2.1, p. 21). As a growth vessel, a Bio-Safe Carrier (Nalgene®) was used. It comprises two polycarbonate parts, a lid and a base, held together by six clamps (Fig. 4.1).

The inner dimensions of the container parts are specified in Table 4.6. Since the vessel was made of polycarbonate it was autoclavable. The box should not be closed during the autoclaving in order to prevent stress cracking. The original silicone gaskets were replaced with silicone hoses (d = 6 mm) which fit better into the container's groove. Thereby, the permeability of the vessel could be reduced. The polycarbonate clamps originally attached to the carrier were not stable enough to keep the vessel closed under pressure. So they were replaced by six custom-made aluminum clamps which could be tightened by screws. The medium was supplied by an inlet on the lower border of the base. It was connected

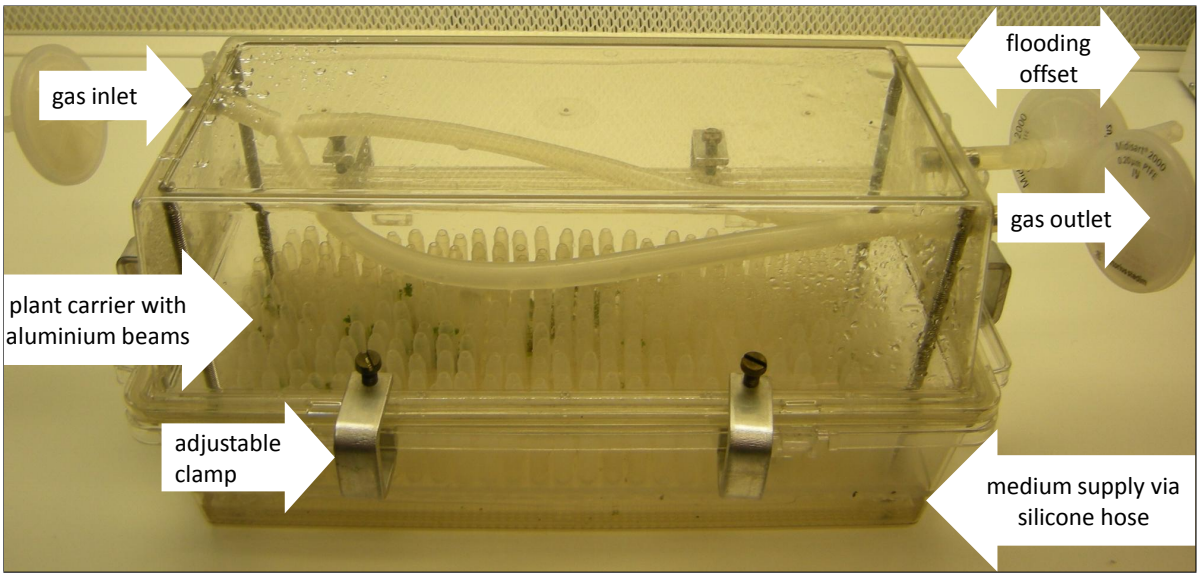


Fig. 4.1: Custom-built temporary immersion system: polycarbonate cultivation box including carrier with plants, gas inlet and outlet, and adjustable clamps

via silicone tubing (d = 10 mm) to a medium bottle (Schott, 2 l). Additionally, another inlet was placed into the lid for the reverse pumping of the medium (see Fig. 4.1).

Tab. 4.6: PA-TIS cultivation vessel dimensions

	Lid (cm)	**Base** (cm)
Length (top)	29.6	31.4
Width (top)	12.6	14.3
Length (bottom)	31.1	30.2
Width (bottom)	13.2	13.1
Height	7.9	6.9
Total volume		6.13 l

A carrier was developed to fixate the plants within the cultivation vessel. In the conventional twin-flask system and the commercially available RITA®-system, the plants are swirled around during the flooding process, resulting in plant clusters. Since one of the objectives of this study was rooting the plants, an interlace of newly developed roots was undesirable. For the construction of the carrier, holes (d = 4 mm) were drilled into a polycarbonate plate (31 x 13 cm). These holes were used to fix polypropylene tubes whose tips had been cut off. The plants could be placed and fixated between those tubes in order to prevent floatation and swirling of the shoots. The tube-tips were removed so the medium could rise through the tubes during the flooding and also as a precaution against the flotation of the carrier. Polycarbonate strips were glued underneath the plate

(a) (b)

Fig. 4.2: PA-TIS carrier layout for two different cultures: a) 55 *Malus* shoots, b) 80 *Kalmia* shoots.

to prevent it from bending under thermic stress. Aluminum beams steadied the carrier against floating during the temporary immersion. For the cultivation of *Malus*, the tubes were grouped to form 55 separate fixing points. Since the *Kalmia* shoots were much smaller, more tubes were added. Both layouts are presented in Fig. 4.2.

The gas exchange was provided by the diagonally opposed inlet and outlet in the lid connected by a perforated silicone hose. The distance of the perforations diminished over the length between inlet and outlet in order to overcome the pressure loss and to provide all plants sufficiently with carbon dioxide.

The openings for medium and gas provision were drilled into the polycarbonate box. Silicone hoses (d = 8 mm) were inserted into the holes and fixed with a metal tube. The inlets were additionally sealed with silicone adhesive. The hoses were fit with sterile filters (Midisart, d = 0.2 µm, Sartorius) in order to ensure a sterile culture.

4.4.2 System setup

Two different setups were used in the testing of the PA-TIS. The first one, shown in Fig. 4.3, only comprised a single vessel. The vessel was directly ventilated in a closed-loop. A pump circulated the air that was enriched with carbon dioxide (Air liquide, Germany) provided from a bottle and separated from the system by a magnetic valve. The air flow and carbon dioxide addition were regulated by air-flow meters (1 l min^{-1}). The connecting tubes from the PA-TIS to the IR-CO_2/H_2O-Analyzer and back consisted of PTFE. In order to ensure a high PPFD, the lighting was applied from two sides and overhead with cool-white fluorescent lamps (Osram Cool Daylight). The lamps could be controlled and lowered separately in order to change the light intensity. A bottom cooling device developed at the BGT (FG Biosystem- und Gartenbautechnik, Leibniz Universität Hannover) was placed under the PA-TIS vessel for the reduction of relative humidity and condensation in the lid. The device consisted of three peltier elements fastened under a copper plate that had exactly the same size as the bottom of the PA-TIS. The elements were furnished with CPU-fans in order to dissipate the heat. The medium reservoir consisted of a bottle (Schott, 2 l) connected by a silicone hose to the cultivation vessel. The flooding was realized as already described in Fig. 2.1, but instead of two pumps, one pump and two three-port valves were used. The gas stream could be directed in two directions by this setup (shown as (3) in Fig. 4.3): once into the bottle, resulting in a replacement of the medium into the cultivation vessel, and back

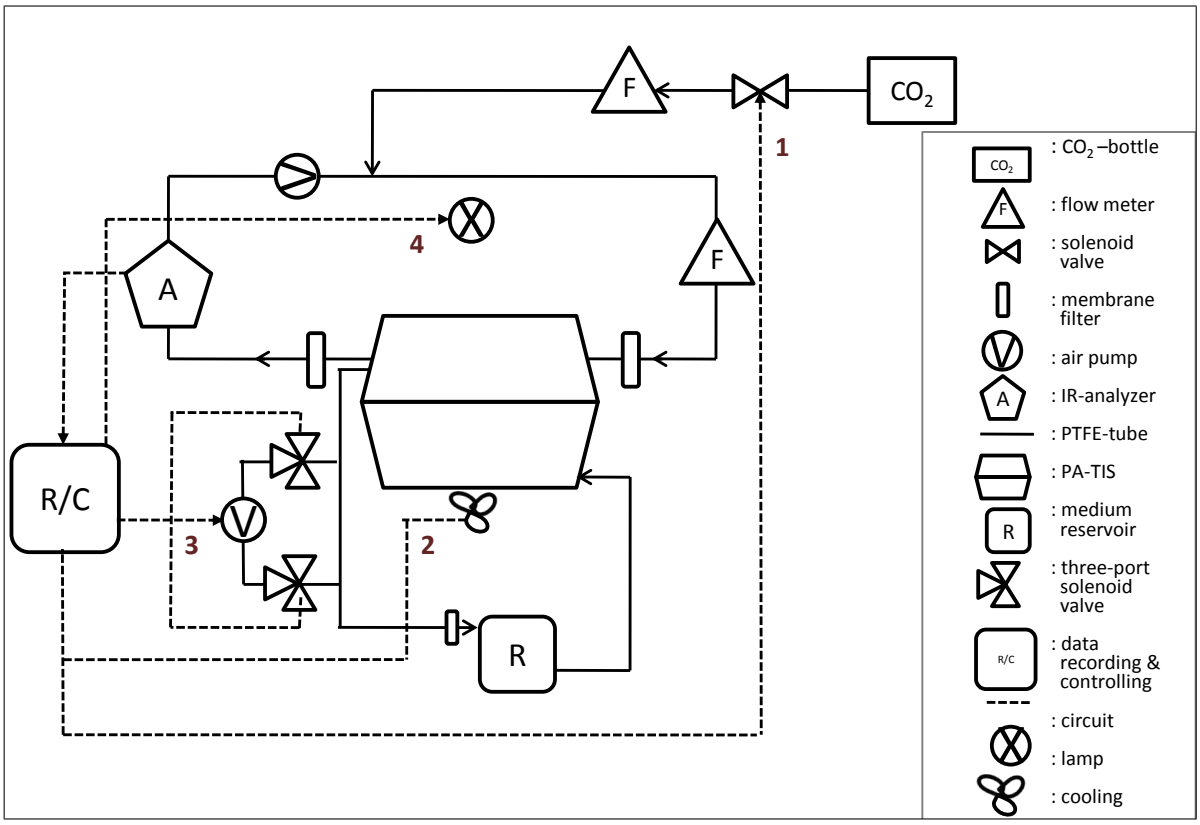


Fig. 4.3: Connection diagram of the first experimental setup. The cultivation air was pumped in a closed circle through the PA-TIS and IR-analyzer (A). If the carbon dioxide concentration was $\leq$ 1200 µmol mol^{-1}, the solenoid valve separating the CO_2-bottle from the PA-TIS was opened (1). Simultaneously, the temperature and relative humidity within the vessel were controlled. If the temperature lay above 24 °C, the bottom cooling was activated (2). The immersion cycle was started every three hours by activating the pump. The three-port valves were used to direct the air flow to the medium reservoir (flooding) or the PA-TIS (pumping back)(3). The lighting (lateral lamps not shown) was kept constantly at 16/8 h light/dark (4).

into the PA-TIS, forcing the medium back into the medium reservoir. The data recording and control of the system was conducted with a data logger system (R/C) developed at the BGT by Udo Düppers (FG Biosystem- und Gartenbautechnik, Leibniz Universität Hannover).

The second setup, depicted in Fig. 4.5, was chosen as a scale-up of the first one in order to increase the plant production threefold. Three PA-TIS vessels were monitored and controlled at the same time. Since only one IR-CO_2/H_2O-Analyzer was available, a measuring gas switchover had to be implemented. The data logger was exchanged with a new recording system, the LabJack U12 (Meilhaus Electronic) and the control was realized over the ME-UBRE relays (Meilhaus Electronic)(Fig. 4.5, U12/UBRE). Three

valves (Fig. 4.5 C1-C3) were connected to the carbon dioxide bottle via FEP-hoses. Each one could be controlled separately. Every vessel had a separate ventilation pump and gas circuit, which could be switched into the measuring section by valves (Fig. 4.5, Nº 1-3). The PTFE-tubes were exchanged for FEP-tubes to reduce the carbon dioxide diffusion. For clarity in Fig. 4.5, some circuits from the U12/UBRE to the different valves were omitted. A photograph of the actual installation of all three vessels is shown in Figure 4.4. In the following chapters, the monitoring and control of the different *in vitro* growth factors will be discussed in detail.

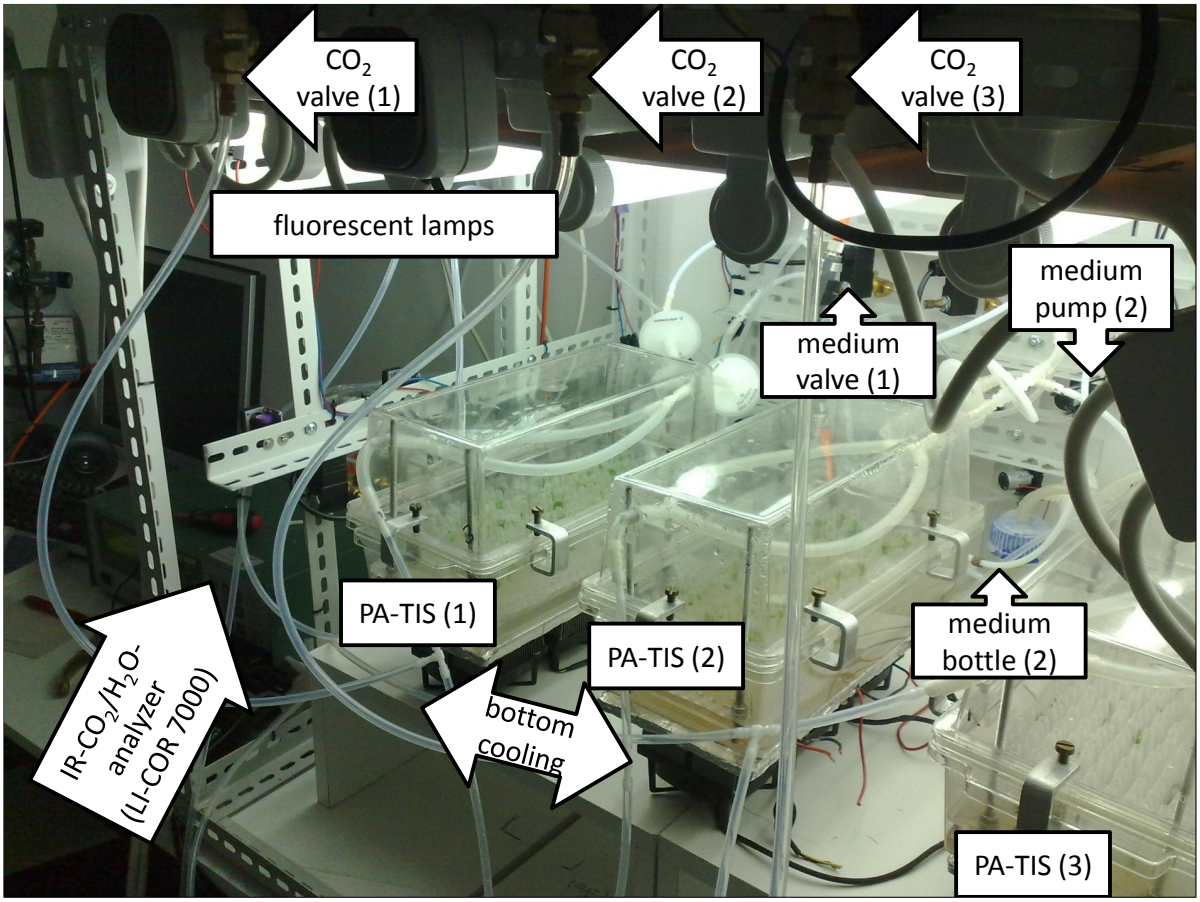


Fig. 4.4: Picture of the second experimental setup (left to right, clockwise): CO_2 valves 1 to 3 (C1-C3 in Fig. 4.5), fluorescent lamps, medium pumps and valves (5 in Fig. 4.5), PA-TIS vessels 1 to 3, bottom cooling and CO_2-analyzer (A).

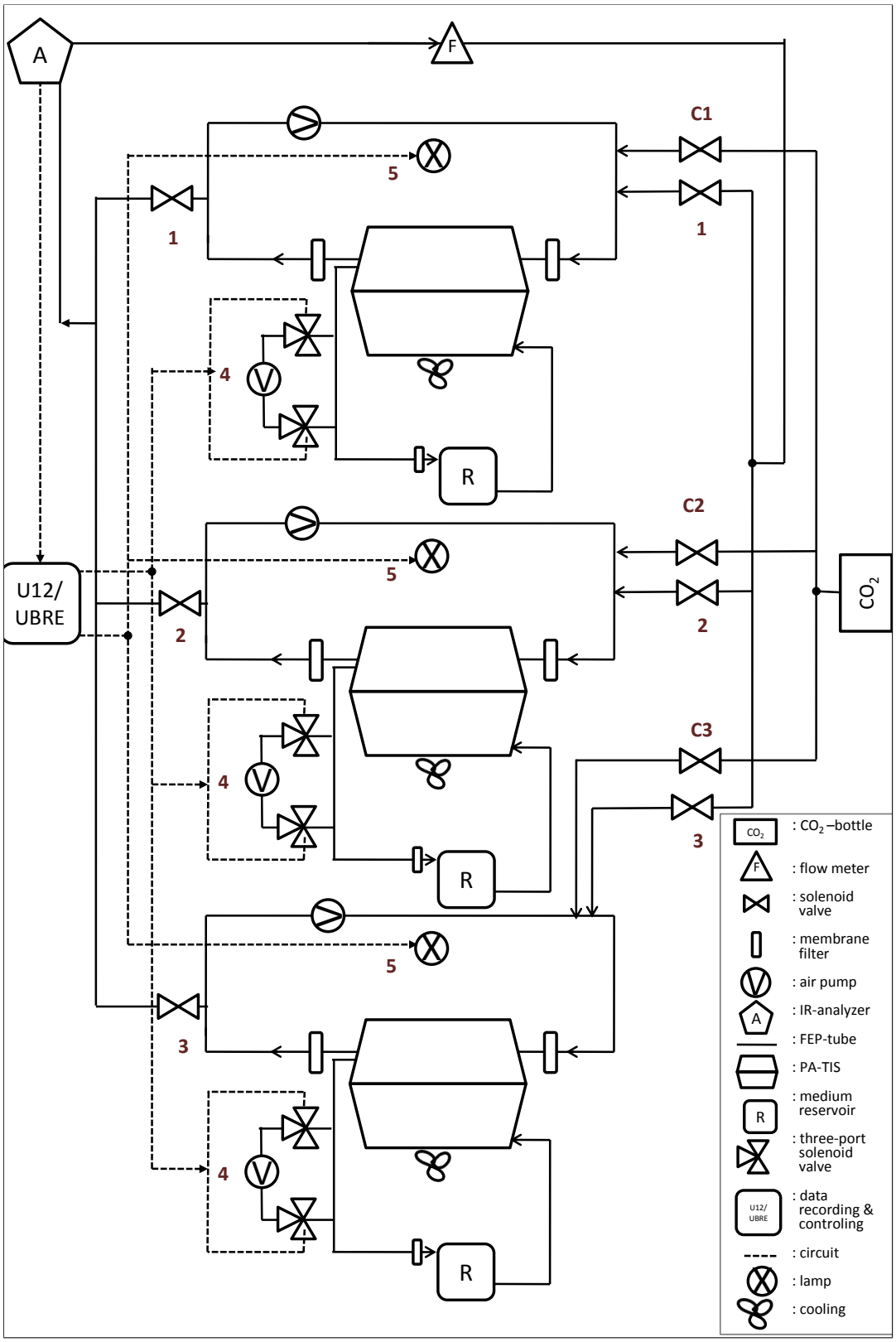


Fig. 4.5: Connection diagram of the second experimental setup. Three PA-TIS vessels were monitored successively with an IR-analyzer (A). The cultivation air was pumped in a closed circle for each vessel separately. The U12/UBRE switched the gas stream to (A) every 10 min (1 - 3; circuits to U12/UBRE are omitted for clarity). If the carbon dioxide concentration was $\leq$ 1200 µmol mol^{-1}, the corresponding solenoid valve to the CO_2-bottle was opened (C1 - C3, circuits to U12/UBRE are omitted for clarity). The immersion cycle was started for all vessels every three hours by activating the pumps. The three-port valves were used to direct the air flow to the medium reservoir (flooding) or the PA-TIS (pumping back)(4). The lighting was either 16/8 h light/dark or 24 h day. The bottom cooling was always activated.

4.4.3 Carbon dioxide and ventilation

The regulation of the carbon dioxide concentration within the PA-TIS was the most critical growth factor for the photoautotrophic culture. The loop, in which the gas was circulated, was closed in both setups (Fig. 4.3 and 4.5, pp. 45 and 47). Since the gas was not enriched externally, carbon dioxide had to be added directly to the PA-TIS during the cultivation. In order to ensure a constant CO_2-supply for photosynthesis, carbon dioxide was provided whenever a concentration of 1200 µmol mol^{-1} was undercut. Carbon dioxide was added in a short pulse; the first measurement after the addition was ignored, because the gas first had to be dispersed and equally distributed within the system (response to a Dirac pulse). The carbon dioxide concentration in the vessel decreased by the photosynthetic activity of the plants as well as leakage and diffusion of the vessel material and connecting hoses.

The IR-analyzer was connected to the measuring system or LabJack via four DAC channels. The raw signal ranged from 0 to 5 V, which could be calibrated to the wanted concentration range (0 - 3000 µmol mol^{-1}). In order to convert the output signal in volts (U_{out}) to µmol mol^{-1}, the following equation was used:

$$c_{CO_2} = (c_{CO_{2max}} - c_{CO_{2_{min}}}) \cdot \frac{U_{out}}{U_{max}} + c_{CO_{2_{min}}} \tag{4.1}$$

where $c_{CO_{2max}}$ is the highest carbon dioxide concentration to be measured (here: first setup 2000 µmol mol^{-1}, second setup 3000 µmol mol^{-1}) and $c_{CO_{2_{min}}}$ the lowest (here: 0 µmol mol^{-1}). U_{max} was 5 V. Before each run, a two-point calibration was carried out. The zero point was adjusted with nitrogen from a bottle (Air liquide) that was scrubbed from any impurities through a chemical filter containing soda lime (carbon dioxide retention) and magnesium perchlorate (water removal). The second point was adjusted with a calibration gas (Air liquide, 1474 ± 28 µmol mol^{-1}).

Although the IR-analyzer provided an accuracy of 1 % within the range of 0 - 3000 µmol mol^{-1} , the calibration gas provided was less accurate (± 2 %). It can be assumed that while the measurements themselves may have differed by up to 2 % from the actual concentration, the absolute differences determined for the CO_2-consumption still were accurate. For security reasons, the carbon dioxide concentration of the cultivation room was monitored continuously. In case of a carbon dioxide leak and elevated carbon dioxide room-concentration (> 2000 µmol mol^{-1}), an acoustic warning signal was implemented.

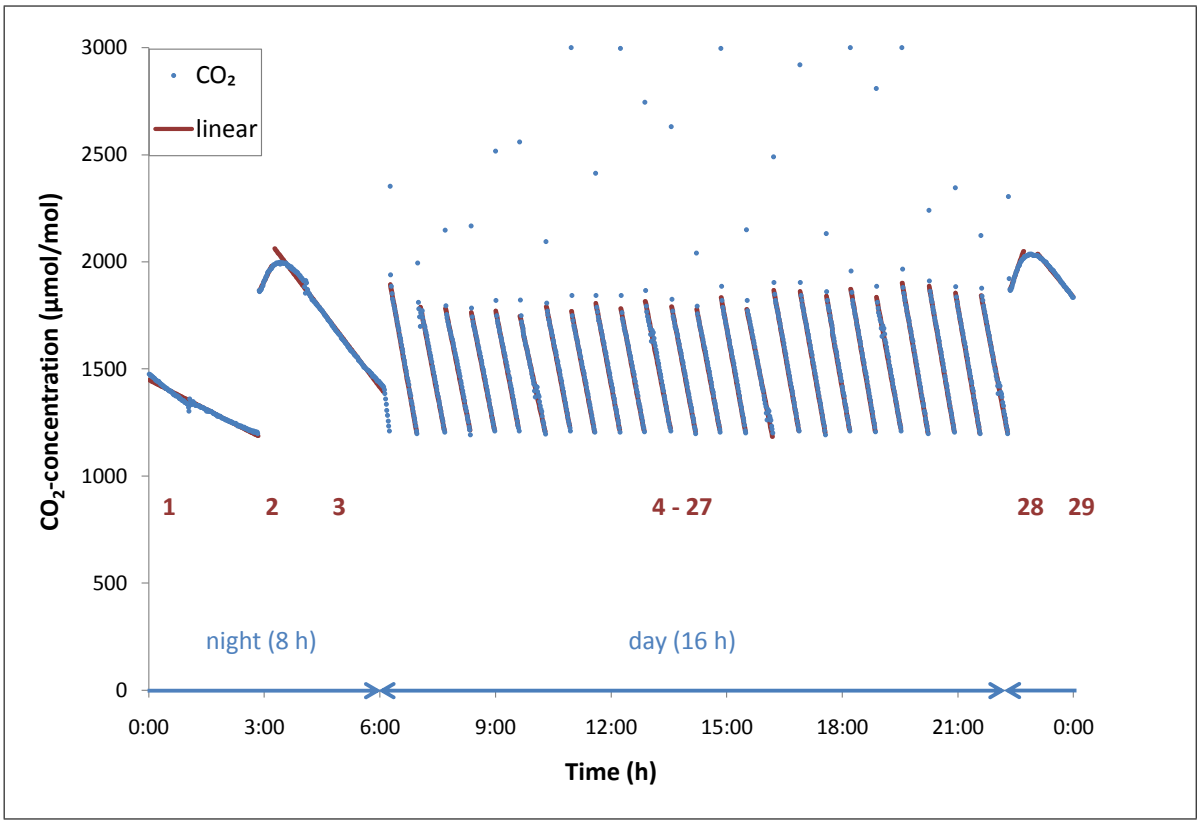


Fig. 4.6: CO_2-concentration in a PA-TIS vessel (second setup) when only one vessel is controlled. Division into 29 sections that were analyzed by exponential and linear regression (Blue = measurement; red = linear regression).

In Fig. 4.6, a typical course of the carbon dioxide concentration within PA-TIS during the cultivation of 'Holsteiner Cox' is depicted (single vessel regulated with the LabJack system without gas-switching). The day was divided into different time periods, depending on the run of the CO_2-curve. In this case, it was split into 29 partitions. For each partition, the increase or decrease in the carbon dioxide concentration was calculated by regression. Normally, the decrease of carbon dioxide in a permeable vessel would be assumed to be exponential (Sallanon et al., 1997):

$$c_{in}(t) - c_{out} = Ae^{-\alpha t} \tag{4.2}$$

where C_{in} and C_{out} are the carbon dioxide concentrations inside and outside of the cultivation vessel, respectively, and t is the time. A is a constant determined from the initial condition C_{in}(t=0)-C_{out} and α is the slope. For the aforementioned time periods, $C_{in}(t) - C_{out}$ (recorded as "CO2ist" and "Vai1" with the LabJack) was determined and an exponential regression of each partition (1 through 29) was carried out with Excel 2007. The results of the regression are listed in Tab. A.6 (p. 180). During the day, the

slope α was fairly constant: α= 0.0143 ± 0.0008. A was also quite stable at 1526 ± 63 µmol mol^{-1}. Hence it could be presumed that the decrease of CO_2 could also be approximated by linear regression.

$$c_{in}(t) = \Delta c_{CO_2} t + c_{in}(t = 0) \tag{4.3}$$

The decrease of carbon dioxide, Δc_{CO_2} (µmol mol^{-1} min^{-1}), is the linear slope. The linear regression for the same periods of time (1 through 29) is shown in Tab. A.7 (p. 181). The arithmetic mean of all slopes is -16.0 ± 1.0 µmol mol^{-1} min^{-1}. Due to the fact that such a graphical approach is time-consuming and a lot of data was accumulated during the cultivation of 'Holsteiner Cox' (28 days) and *Kalmia latifolia* (42 days), a simpler way of analysis had to be found. Since the decrease was shown to be linear, a simple subtraction between two measuring points was chosen as approximation for Δc_{CO_2}.

$$\Delta c_{CO_2} = \frac{c_{CO_2}(2) - c_{CO_2}(1)}{t(2) - t(1)} \tag{4.4}$$

The mean value for all differences from 06:00 to 22:00 was -16.5 ± 4.1 µmol mol^{-1} min^{-1}, differing only little from the value attained by linear regression (0.5 µmol mol^{-1} min^{-1}, below the measurement sensitivity). This approach proved to be quite effective for the second setup that featured more than one vessel (see Fig. 4.5, p. 47). Each vessel could only be monitored for 10 minutes at a time, resulting in an interrupted measurement such as presented in Fig. 4.7.

Herein, the division into different periods is not as easy as before, because the measurement is discontinuous and a trend is hard to detect. A linear regression is hence difficult. The results of such a regression are given in Tab. A.5 (p. 179). For the same day, the results for Δc_{CO_2} according to Eq. 4.4 averaged -8.3 µmol mol^{-1} min^{-1} whereas the result of the linear regression was -7.8 ± 1.6 µmol mol^{-1} min^{-1}. The difference of 0.5 µmol mol^{-1} min^{-1} can be explained by some values that were not included in the linear regression, because there were too few data points to plot (e.g. between sections 16 and 17).

The course during the dark period was more complicated, because the production of CO_2 through respiration resulted in an increase of CO_2, whereas the vessel leakage led to a reduced carbon dioxide concentration. In the sections 2 and 3 of Fig. 4.6 and the sections 1 and 2 of Fig 4.7, these opposing effects are apparent: the concentration within the vessel rises up to a certain point where the influence of the leakage is higher than the respiration rate. As a result, the concentration drops again. The loss of CO_2 that will be

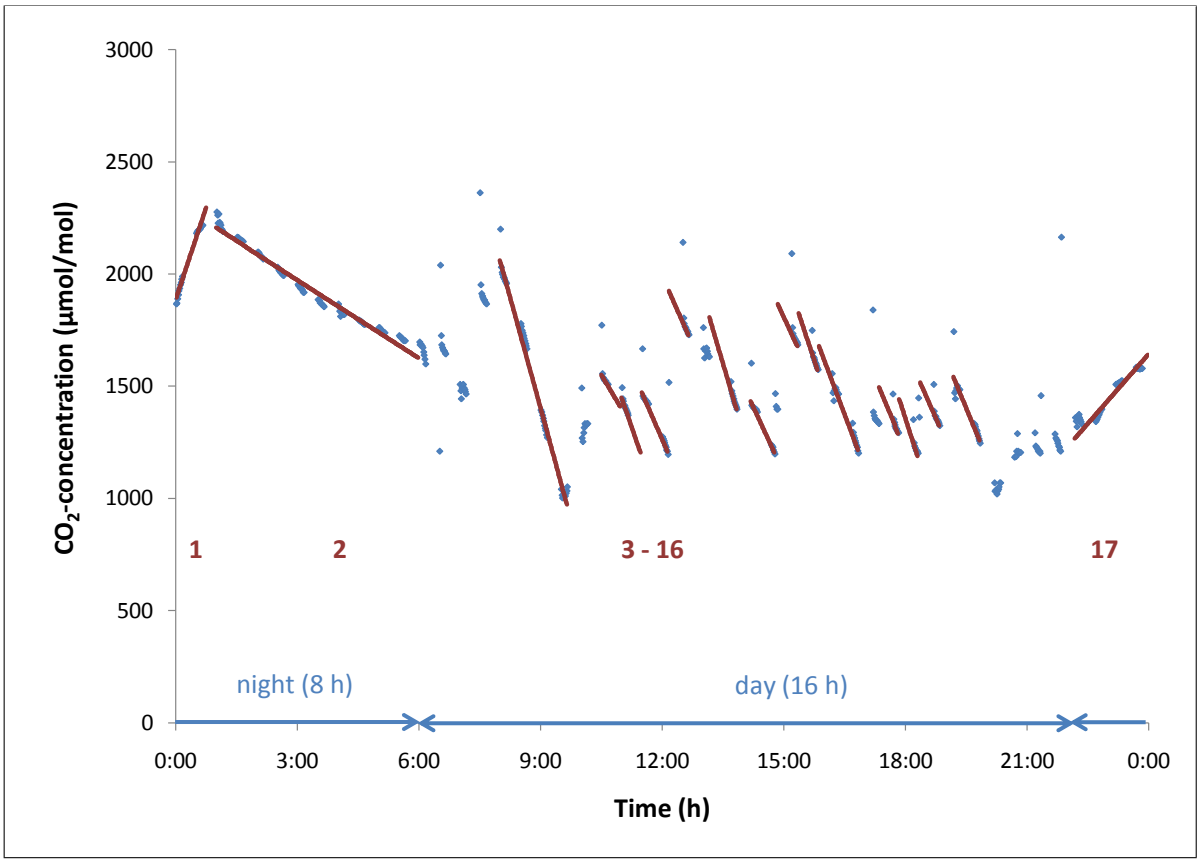


Fig. 4.7: CO_2-concentration in a PA-TIS vessel (second setup) when three vessels are controlled. Division into 17 sections that were analyzed by linear regression (Blue = measurement; red = linear regression).

discussed in the following was included in the calculations of net photosynthetic rate and respiration (both Eq. 4.5, p. 54).

It can be concluded that even though the carbon dioxide decrease is exponential, it is safe to approximate it linearly, since the concentration never falls below 1200 µmol mol^{-1} and the linear region is not left. From the carbon dioxide progression in Fig. 4.6 and 4.7, it can be deduced that the slope during the night is much lower than during the day. The difference between Δc_{CO_2} (day) and Δc_{CO_2} (night) has to result from the plants' photosynthetic activity. As described in equation 4.5, the net photosynthetic rate can be calculated if the carbon dioxide uptake and loss is known. In period 27 in Tab. A.7 (p. 181) and period 1 and 17 in Tab. A.5 (p. 179), an increase of CO_2 can be stated. Since it takes place in the darkness and is not a result of carbon dioxide addition to the system, it may be concluded that the CO_2 is produced by dark respiration. As suggested by Sallanon et al. (1997), the photorespiratory activity during the day was neglected when calculating the net photosynthetic rate. The P_n-results for the cultivations of

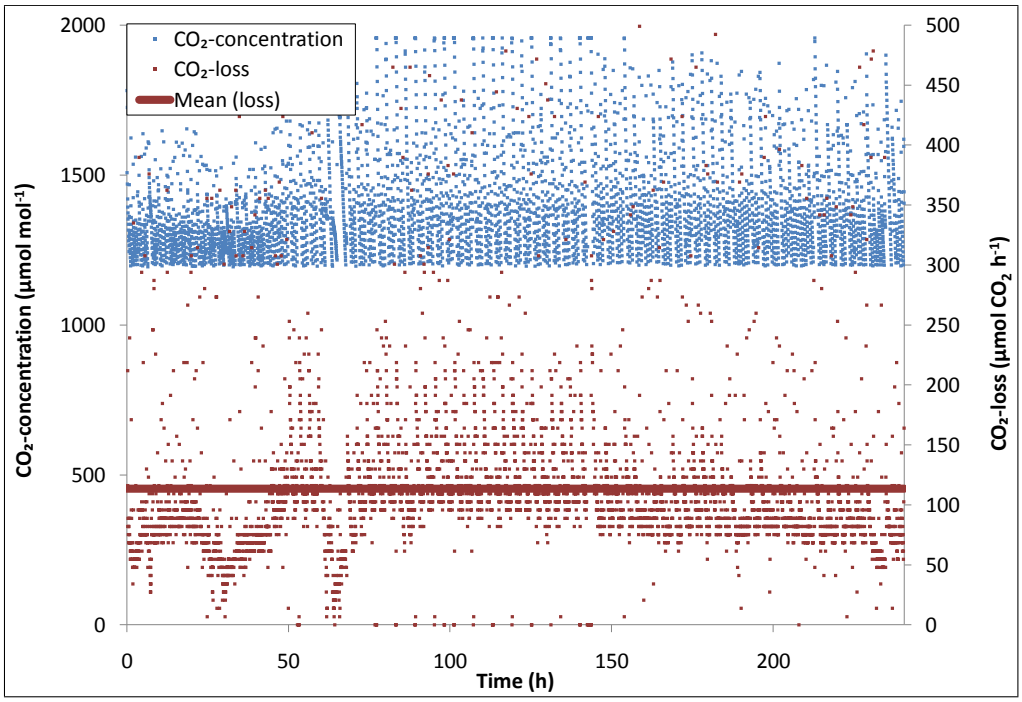
'Holsteiner Cox' and *Kalmia latifolia* can be found in the respective Chapters 5.2.2 and 5.3.2.

Adding gas to a closed system will result in bursting the vessel. Therefore, the vessel could not be totally sealed, which resulted in a certain amount of gas leaking through the gasket when the pressure in the vessel was too high. The carbon dioxide concentration within the respective empty PA-TIS vessels was recorded individually for 10 days and the loss calculated with Eq. 4.4 (p. 50). The results for both setups are shown in Fig. 4.8.

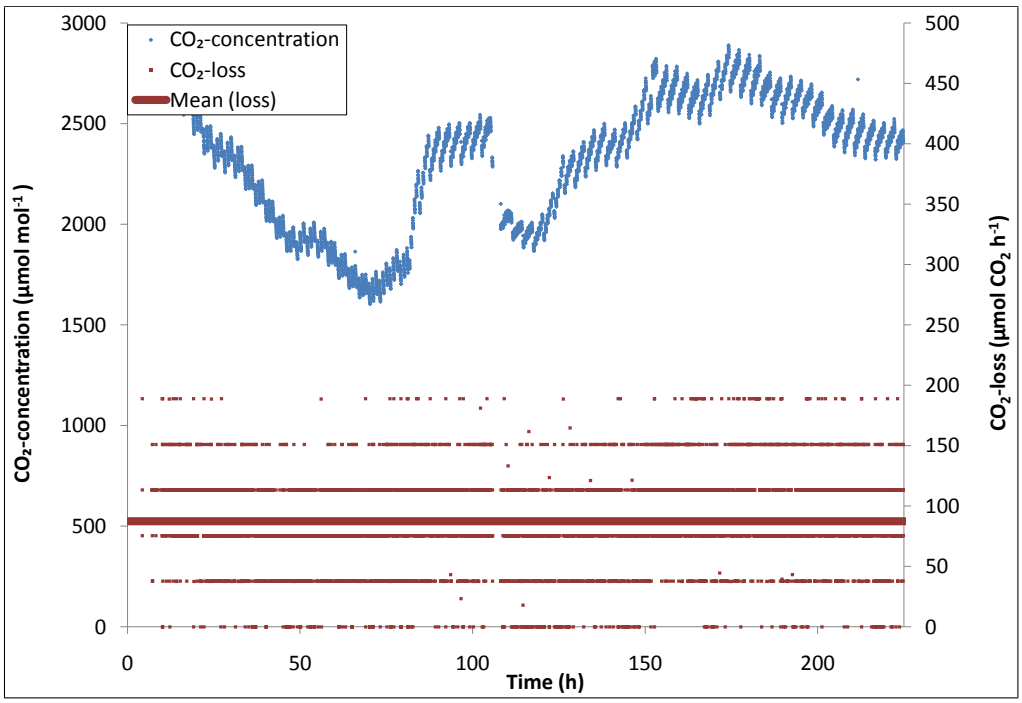
For the first setup (Fig. 4.8a), the variation of the carbon dioxide loss was quite high, with a leakage of up to 500 µmol h^{-1}. The mean loss was determined to be 114 µmol h^{-1}. The loss in the second setup was lower due to the changes of material for the tubing and shorter connections to the IR-analyzer. Here, the mean leakage of the vessel was 87 µmol h^{-1}. These values were used as the term $\frac{\Delta c_{loss}}{\Delta t}$ in Eq. 4.5 (p. 54) for the determination of P_n.

If one compares the Figures 4.8a and 4.8b, it becomes obvious that the first setup only allowed a measurement of CO_2 between 0 and 2000 µmol mol^{-1} whereas concentrations of up to 3000 µmol mol^{-1} could be determined in the second setup. Also, the recording interval between two measurements was longer, measurements were only taken every 3 to 5 minutes. In the second setup then again, three vessels were controlled for 10 minutes each, but with an interval of only one minute between the data recordings. The gas-switch between the three vessels occurred without flushing the IR-analyzer. This resulted in a slight gas-intermixture between the different vessels. The influence of one vessel on another becomes obvious when the carbon dioxide concentration difference between two vessels was high, like in Fig. 4.8b. Due to a high carbon dioxide concentration in the antecedent vessel, the concentration rose between the end of a measuring cycle and the beginning of a new one. This influence was neglected by discarding the differences between the end of an old and the beginning of a new measurement cycle.

All courses of the carbon dioxide concentration within the PA-TIS vessels are shown in Tab. 5.1 to 5.4 (pp. 63). Since Excel 2007 cannot display more than 32000 values in one Figure, only the first 22 days of the cultivation could be presented for the second setup (Tab. 5.3 and 5.4).



(a)



(b)

Fig. 4.8: CO_2-concentration (µmol mol^{-1}) and loss (µmol h^{-1}) of an empty PA-TIS vessel a) in the first setup; b) in the second setup. (Blue = carbon dioxide concentration; red = carbon dioxide loss).

4.4.4 Net photosynthetic rate

Zobayed et al. (1999b) developed an equation similar to Fujiwara et al. (1988)(see Eq. 2.1 and 2.2, p. 24) for the estimation of the net photosynthetic rate (P_n) *in vitro*. Both equations apply to open-ventilation methods, so that Eq. 2.2 was modified for the PA-TIS in order to describe the closed-loop system used here.

$$P_n = -\frac{V}{n} \cdot \left(\frac{\Delta c_{uptake}}{\Delta t} - \frac{\Delta c_{loss}}{\Delta t}\right) \cdot \frac{\rho_{air}}{M_{air}} \tag{4.5}$$

The dimension of P_n is [µmol CO_2 h^{-1} $plant^{-1}$]. V is the volume of the whole closed system including the vessel and the connecting tubes. The volumes for both setups (Fig. 4.3 and 4.5) are listed in Table 4.7. n is the number of plants contained within the box. The carbon dioxide uptake Δc_{uptake} of carbon dioxide (µmol mol^{-1}) was estimated between two measurements (time difference Δt in h). The loss of carbon dioxide by the empty vessel was included by c_{loss}. The molar mass of air, M_{air}, equals 28.9644 g mol^{-1}. The air density ρ_{air} was assumed to be 1.184 g m^{-3} for 25 °C and 101.325 kPa. As carbon dioxide was monitored day and night, the respiration during the night was calculated by the same equation, resulting in a negative value [µmol CO_2 h^{-1} $plant^{-1}$], because from the plants' position respiration means a loss of carbon dioxide.

Tab. 4.7: PA-TIS head space (total)

	Length (m)	**Radius** (m)	**Volume** (l)
First setup			
PTFE-hoses	8.88	0.002	0.11
Silicone-hoses	0.56	0.005	0.04
Cultivation vessel			6.13
TOTAL			6.29
Second setup			
FEP-hoses	6.895	0.02	0.09
Silicone hoses	0.56	0.005	0.04
Cultivation vessel			6.13
TOTAL			6.26

4.4.5 PA-TIS immersion cycles

The medium was divided during the setting of the plants. The medium reservoir contained 2 l of rooting medium (MS or WPM), whereas 0.5 l were poured into the cultivation vessel. Thereby, a total immersion of the plants during flooding was ensured, especially of the larger 'Holsteiner Cox' shoots. The immersion cycles could be varied. The time for pumping the medium into the vessel and back into the medium bottle could be regulated separately. The immersion cycles were adjusted to a complete flooding of the shoots for 6 minutes every 3 hours. The pump cycles started at 01:00 whereupon the medium was pumped from the reservoir into the PA-TIS. At 01:04 the medium was pumped back into the medium bottle with the cycle ending at 01:08. The plants were in contact with the medium for 6 minutes. The complete pumping cycle took 2 minutes longer, since the medium had to reach the plants first.

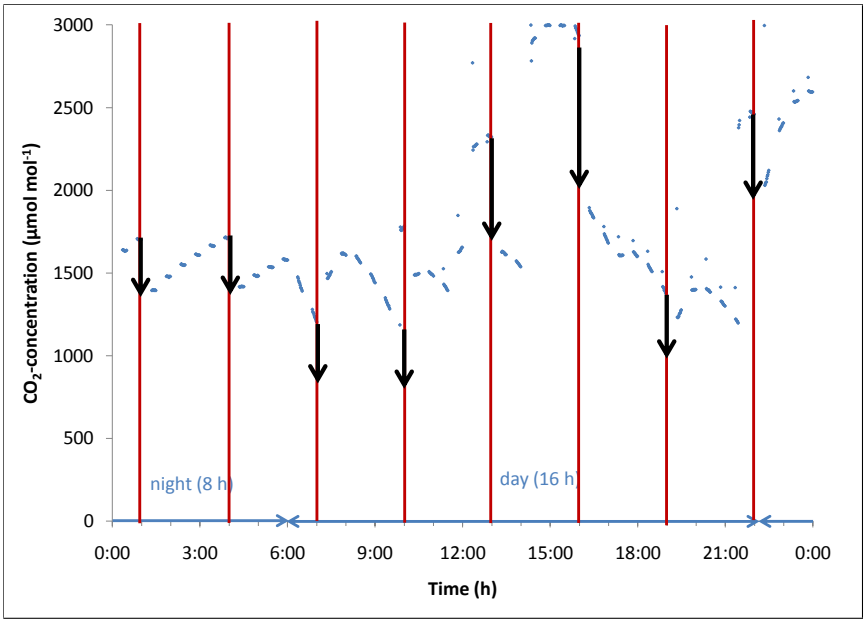


Fig. 4.9: Influence of the temporary immersion cycles on the carbon dioxide concentration within the PA-TIS. The start of the flooding process is indicated by the red lines and the loss of carbon dioxide by the black arrows.

The transport of the medium from the reservoir to the plants was only possible through displacement of air. This displacement resulted in a pressure change within the PA-TIS, which had an influence on the carbon dioxide concentration, resulting in a higher loss of CO_2 during the cycles.

In Fig. 4.9, the start of the temporary immersion is marked with red lines. After each cycle, the concentration within the vessel was much lower (indicated by the arrows). This

carbon dioxide drop was not a result of photosynthetic activity of the plants; hence these values were neglected in the calculation of P_n.

4.4.6 Relative humidity

The water content (µmol mol^{-1}) of the gas within the vessels was measured with the same IR-analyzer as the carbon dioxide. In order to determine the relative humidity (rH, %), the water content had first to be converted to the specific water content x (g kg^{-1}).

$$x = \frac{n_{H2O}}{n_{air}} \cdot \frac{M_{H2O}}{M_{air}} \tag{4.6}$$

x could then be converted to rH, if the temperature and pressure within the vessel were known. Both were recorded simultaneously with the carbon dioxide concentration and water content. The conversion was performed with the help of a Visual Basic plug-in for Excel, kindly provided by Dr. Burkhard von Elsner (FG Biosystem- und Gartenbautechnik, Leibniz Universität Hannover).

4.4.7 Lighting

The lighting was varied in the tests with 'Holsteiner Cox'. The PPFD was measured with a PAR-sensor (LI-190, LI-COR) inside the empty PA-TIS vessels at the actual cultivation height and inside an empty plastic cup for the reference cultures. At first, the PA-TIS was lighted laterally (2 sides, 115 µmol m^{-2} s^{-1}) and overhead by cool-white fluorescent lamps (75 µmol m^{-2} s^{-1}). Later, the overhead lighting was installed closer to the PA-TIS in order to provide a higher PPFD (100 µmol m^{-2} s^{-1}). Since the lateral lighting had a high influence on the rooting efficiency and plant growth, only overhead lighting was installed the second with a high PPFD of 150 µmol m^{-2} s^{-1} as shown in Fig. 4.4 for tests on 'Holsteiner Cox' as well as *Kalmia latifolia*. Throughout literature (Tab. A.3, p. 178), photoautotrophic cultures were always tested against photomixotrophic reference cultures. These cultures were always kept under 'normal' conditions, meaning that ventilation and PPFD were not increased. In the present study, the reference cultures on agar medium were always cultivated under 75 µmol m^{-2} s^{-1} since this PPFD was also measured in the cultivation rooms of the cell culture laboratory were 'Holsteiner Cox' and *Kalmia latifolia* were normally cultivated. The light/dark cycle was adjusted to a 16 h

day and an 8 h night period (for *Malus* and *Kalmia* shoots) both in the first setup or continuous lighting (24 h) only for *Malus* in the second setup.

4.4.8 Temperature

The first tests were conducted in a room that was not air-conditioned, which affected the cultivation temperature significantly. A bottom cooling system, described in Chapter 4.4.2, was provided in order to lower the dew point temperature within the PA-TIS vessel and prevent unwanted condensation of water in the tubing or on the vessel lid. The only possibility to reduce the vessel temperature was by regulating the bottom cooling, but the temperature reduction was limited by the peltier elements' cooling capacity.

Later in the second setup, the room temperature was regulated by an air-conditioning system. The bottom cooling was kept continuously at 18.5 °C. The room temperature had a direct influence on the vessel temperature. Depending on the culture, the room temperature was adjusted. The room temperature was controlled by a NTC-thermistor (NTC20k) placed between the plastic cups.

4.4.9 Data acquisition and regulation of growth factors

The first tests were conducted with a data logger and controlling system developed at the BGT (FG Biosystem- und Gartenbautechnik, Leibniz Universität Hannover) by Udo Düppers. Since the corresponding data logger only provided limited data storage space (64 kB) and more than one PA-TIS vessel had to be regulated (scale-up of the first setup to the second setup (Fig. 4.5)), another method of data acquisition was chosen. Given that only one IR-analyzer was available, only one vessel could be monitored at a time. The gas had to be switched from the standby-loop to the measuring loop. Each vessel was measured for 10 minutes before changing to the next, allowing a total measuring time of 20 minutes per vessel and hour. The LabJack U12 (Meilhaus Electronic) was chosen as interface for its user-friendly software ProfiLab. In the following, the respective parts of the regulation and monitoring will be introduced.

The ProfiLab interface for the U12-device is presented in Fig. 4.10. In the upper left corner the timers for the temporary immersion are depicted. The timer-function is useful for controlling pumps/lighting etc. in defined periods of time that do not depend on measurements. In this case, the flooding was regulated by turning on the pump (DT_1)

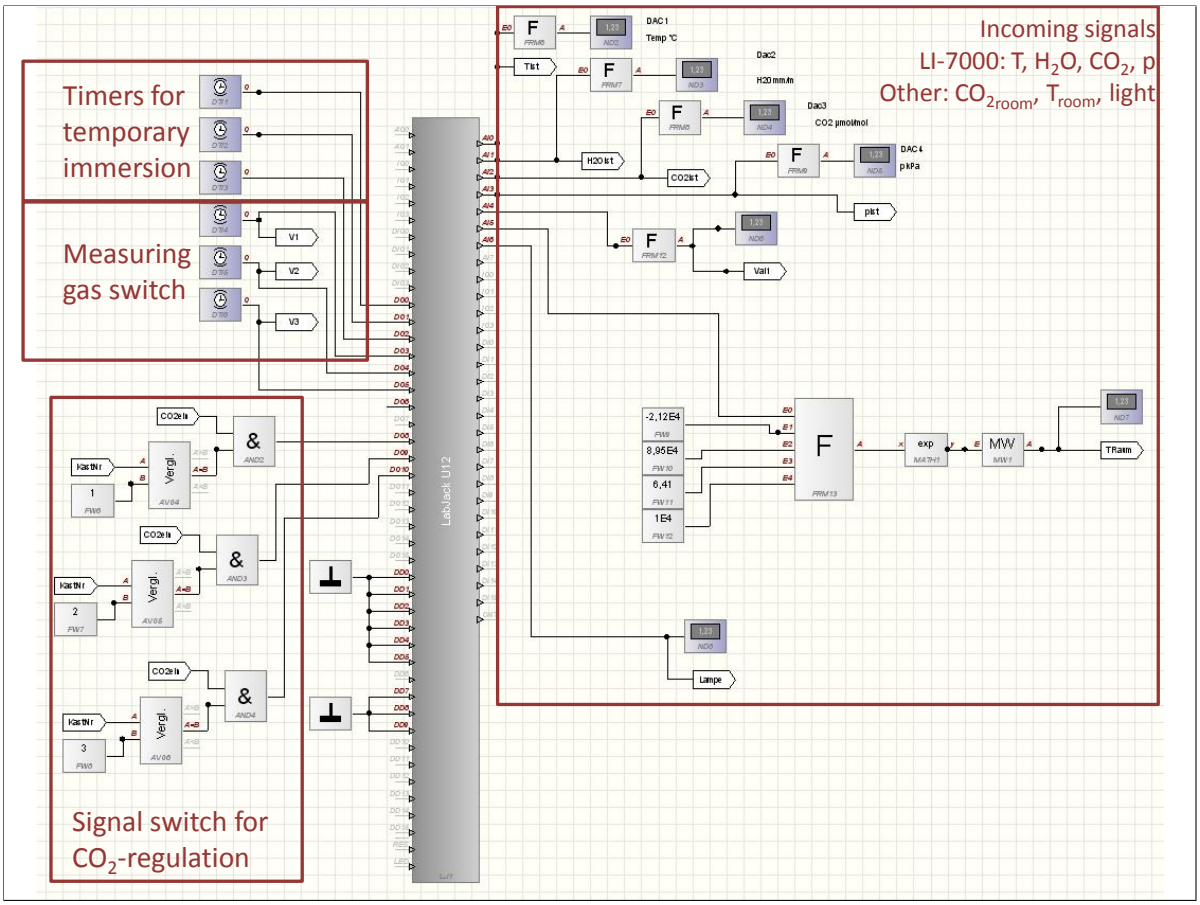


Fig. 4.10: Connection diagram under ProfiLab for U12-device: input signals, TI-timer, gas-switch, CO_2-regulation

and the valves directing the air into the medium bottle. Later, the flow was directed in the opposite direction by turning on the second set of valves, resulting in draining the system. Directly below, the gas-switching described in Chapter 4.4.2 was implemented. Every 10 minutes the circuit to the IR-analyzer was changed by switching on the valves connecting the respective vessel to the measuring circuit. Since the circuits were always closed, a slight overlap between the vessels' gas occurred within the first minute of measurement. That is why those values were excluded in later analyses. On the upper right side the incoming signals from the IR-analyzer (LI-7000) and other devices are shown. The data are symbolized as arrows, the so-called jumpers.

In order to describe the regulation of the carbon dioxide concentration within the PA-TIS shown in the lower left side of Fig. 4.10, a further part of the regulation depicted in Fig. 4.11 has to be taken into consideration. The CO_2-jumper for the signal coming from the IR-analyzer was called "CO2ist". The CO_2-setpoint is defined by "CO2sol". In all present tests, the setpoint was 1200 µmol mol^{-1}. As seen in Fig. 4.11, "COist"

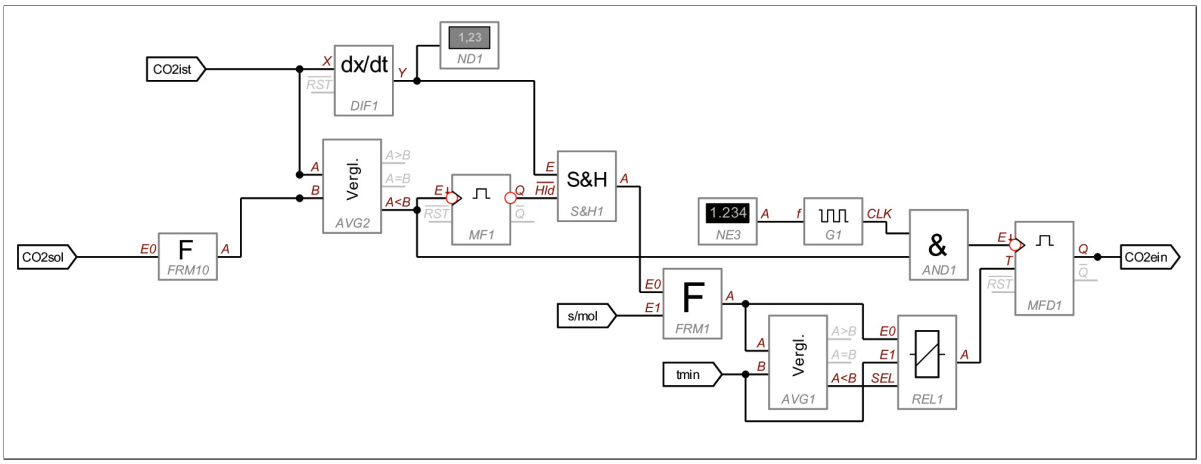
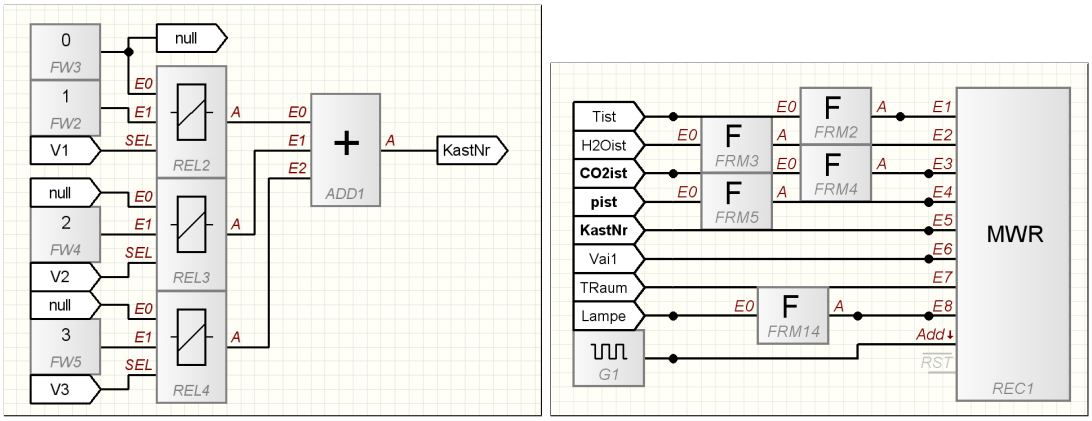


Fig. 4.11: CO_2-regulation programmed under ProfiLab: Input signal (CO2ist), comparison to set-point (CO2sol), signal to relay (CO2ein)

was compared to "CO2sol". If the concentration within the PA-TIS was lower than the setpoint (A<B), the valve connecting the CO_2-bottle to the system was activated (as shown in Fig. 4.3 and 4.5).



(a) (b)

Fig. 4.12: (a) Identification of PA-TIS vessel to be regulated under ProfiLab. (b)Data acquisition under ProfiLab: incoming data and data recorder (MWR)

The opening time was correlated with the difference (CO2sol-COist). The bigger the difference, the longer the valve was opened ("CO2ein" defined in milliseconds). The correlation factor could be chosen by the entering "s/mol". Thereby, the amount of CO_2 added to the PA-TIS vessels could be directly influenced. Since the net photosynthesis rate was calculated by the decrease of carbon dioxide over time, a high amount of CO_2 was added, resulting in a sawtooth wave (see Fig. 4.6, p. 49). The addition of carbon dioxide could of course be kept minimal by changing "s/mol" so that the concentration

would be constant, but in the present test the sawtooth wave was necessary for the determination of P_n.

To supply the right vessel with carbon dioxide at the right time, a vessel identification was programmed (Fig. 4.12a). If the vessel number "KastNr" was known and "CO2ein" was larger than zero (ms), carbon dioxide was added to the respective vessel. In such a manner, other factors could also be regulated (e.g. bottom cooling), but they were kept constant in the second setup.

The final data acquisition for all measured values is shown in Fig. 4.12b. The following data were recorded:

- Tist = temperature in the PA-TIS [°C]
- H2Oist = water content of the gas in the PA-TIS [mmol mol^{-1}]
- CO2ist = carbon dioxide concentration in the PA-TIS [µmol mol^{-1}]
- pist = air pressure in the PA-TIS [kPa]
- KastNr = vessel currently monitored [1-3]
- Vai1 = carbon dioxide concentration in the cultivation room [µmol mol^{-1}]
- Lampe = light on/off [1/0]
- G1 = time intervals between two measurements [min]

The measurements were taken every minute and recorded in a txt-file, which was later analyzed in Excel 2007.

4.5 Data and statistical analysis

All results of the environmental control were collected continuously and evaluated in Excel 2007.

The statistical tests and boxplots were implemented with SigmaPlot 11.0 (Systat Software). For the statistical evaluation of the shoot weight (fresh and dry weight), leaves (number and length) as well as the roots (number and length), the different runs under the same conditions (see Tab. 4.3 and 4.5) were first compared to each other. When no significant differences were found, the values were pooled (e.g. fresh weight for PA-TIS under 115 + 100 µmol m^{-2} s^{-1}). When significant differences between the runs occurred, they were presented individually.

In order to compare two runs or treatments, a determination whether the probes were normally distributed had to be executed beforehand. Therefore, a Shapiro-Wilk test was conducted. When the probes passed the normality test, a Student-Newman-Keuls-test was used for the pairwise comparison, otherwise a Mann-Whitney rank sum test was chosen.

A pairwise comparison of more than two treatments, such as the reference cultures and the PA-TIS, could only be conducted with help of Dunn's method (Kruskal-Wallis One Way Analysis of Variance on Ranks) since the probes mostly did not have the same size. Different letters were used to label treatments whose mean values differed at $p \leq 0.05$. The results of the analyses are summarized in Chapter A.1.6.

The mean values (arithmetic mean = AM) and standard deviations (SD) for each treatment were calculated, so that every single shoot was treated as a repetition. AM and SD as well as linear and exponential regressions were determined in Excel 2007. The exponential regressions of the net photosynthetic rate were computed with SigmaPlot 11.0 (Systat Software).

All photographs of the stomata were analyzed with GIMP (GNU Image Manipulation Program 2.6.5) using the area detection tool.

5 Results

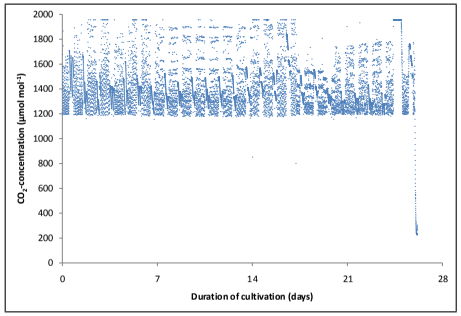
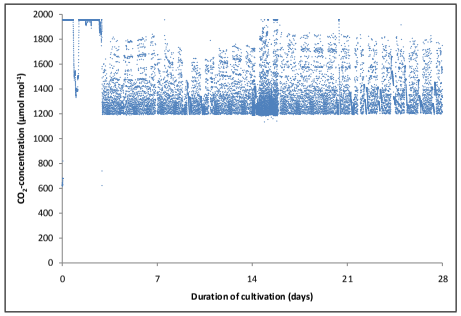
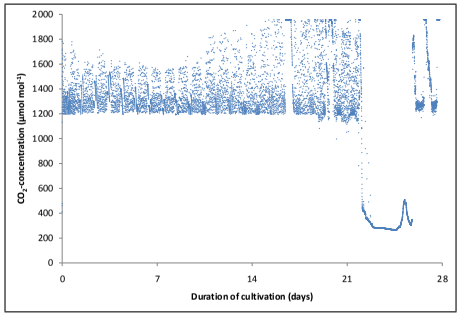
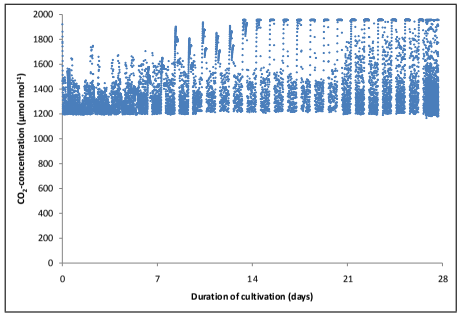
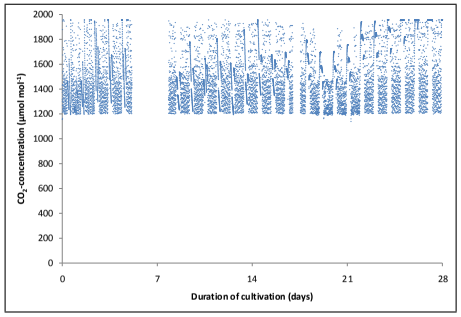
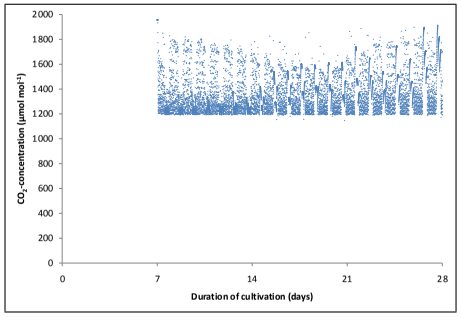
5.1 Results of the cultivation of 'Holsteiner Cox' in the first setup (single PA-TIS vessel)

5.1.1 Carbon dioxide

In the first setup (Fig. 4.3, p. 45), measurements could only be taken every three minutes, because the memory capacity of the data logger was so low (64 kB). Especially in the later days of cultivation, this led to problems in evaluating the carbon dioxide uptake. The first difference measurement (Eq. 4.1, p. 48) after the addition of carbon dioxide was neglected, because the gas first had to disperse within the cultivation vessel. Even with this elimination, the carbon dioxide loss within a vessel in the first setup was much higher than in the second setup (see Fig. 4.8a, p. 53). Throughout the cultivation leaves were produced and biomass accumulated; hence, the plants took up carbon dioxide more quickly in the later days. From one carbon dioxide pulse to the next, only two or three measurements remained to estimate the carbon dioxide uptake. It can thus be assumed that the net photosynthetic rate estimation of P_n by Eq. 4.5 (p. 54) for the first setup is not as accurate as for the second (Fig. 4.3, p. 45).

The results of the carbon dioxide measurements in PA-TIS during the cultivation of 'Holsteiner Cox' shoots are presented in Tab. 5.1. In some of the figures the highest measurable carbon dioxide concentration was reached (1956 µmol mol^{-1}). In this case, the concentration was recorded as 1956 µmol mol^{-1}, but actually surpassed it. Therefore, no carbon dioxide mean value could be determined for the respective cultivations. The lowest concentration in the vessel was normally 1200 µmol mol^{-1}, because carbon dioxide was added as soon as the concentration dropped under this threshold. The course of carbon dioxide was normal in the first two runs under 115 + 75 µmol m^{-2} s^{-1} whereas some problems occurred in the third run (Tab. 5.1c). From the day 23 to 27 of the cultivation, the concentration dropped to approximately 300 µmol mol^{-1}. It did neither rise nor fall for the whole time, so that it can be assumed that the gas stream to the IR-analyzer was blocked. Under 115 + 100 µmol m^{-2} s^{-1} the data for the second and third run are not complete due to a malfunction of the data logger. The net photosynthetic rate could hence not be estimated for these days.

Tab. 5.1: Carbon dioxide within PA-TIS for 'Holsteiner Cox' in the first setup. Left: under 115 + 75 µmol m^{-2} s^{-1} (a,c,e); right: under 115 + 100 µmol m^{-2} s^{-1} (b,d,f). (a+b) first run; (c+d) second run; (e+f) third run

115 + 75 µmol m^{-2} s^{-1}	(a) 	(b) 	(c) 
115 + 100 µmol m^{-2} s^{-1}	(d) 	(e) 	(f) 

5.1.2 Net photosynthetic rate

The net photosynthetic rate for the 'Holsteiner Cox' shoots was calculated according to equation 4.5. The carbon dioxide uptake per minute was recorded during the day and the carbon dioxide production through respiration during the night (see Fig. 4.6 or 4.7). The values for day are represented in green, the values for night in blue. For some days the data sets are not complete, either due to malfunction of the data logger or too high carbon dioxide concentrations that exceeded the measurement range of 2000 µmol mol^{-1} for the first setup or 3000 µmol mol^{-1} for the second setup.

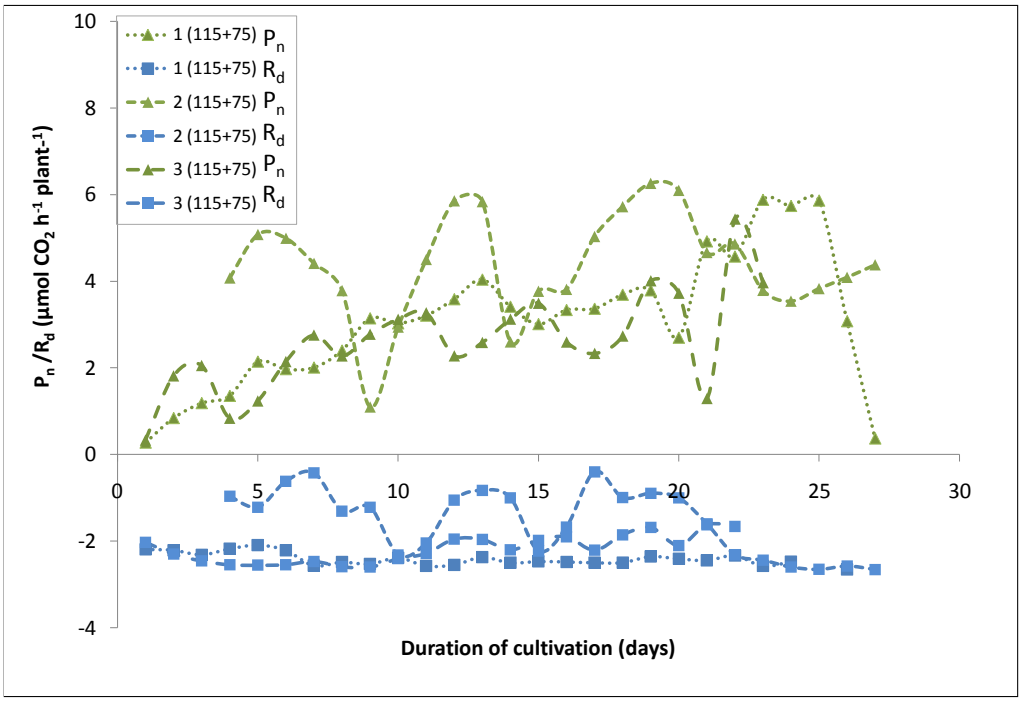


Fig. 5.1: Net photosynthetic rate P_n (day) and dark respiration (night) of the 'Holsteiner Cox' shoots throughout the cultivation in PA-TIS under medium PPFD (115 + 75 µmol m^{-2} s^{-1}, 3 runs.)

Since the photosynthetical uptake of CO_2 and the respiration in the dark are opposed to each other, one is defined as a positive value (P_n) and the other as negative value (R_d). A lower value for R_d therefore corresponds to a rising respiratorial activity and will hence be referred to as "increase". The mean values for all cultivations are recorded in Tab. A.8, p. 182. Cultivations that were not conducted in the same period of time will be called "runs" in the following, whereas cultivations that took place at the same time in the second setup will be called "repetitions".

In Fig. 5.1, the net photosynthetic activity and respiration resulting from the uptake and production of CO_2 during three cultivations of 'Holsteiner Cox' under a PPFD of 115 + 75 µmol m^{-2} s^{-1} are shown. P_n lay between 0.3 and 6.3 µmol h^{-1} $plant^{-1}$. In all cultivations under 115 + 75 µmol m^{-2} s^{-1}, P_n had an upward trend throughout the cultivation, but in the first run it dropped in the last two days of cultivation. The respiration in the dark period (R_d) did not surpass -2.7 µmol h^{-1} $plant^{-1}$ on the last day of cultivation in the first run. R_d did not vary strongly over the course of cultivation, averaging 2 µmol h^{-1} $plant^{-1}$.

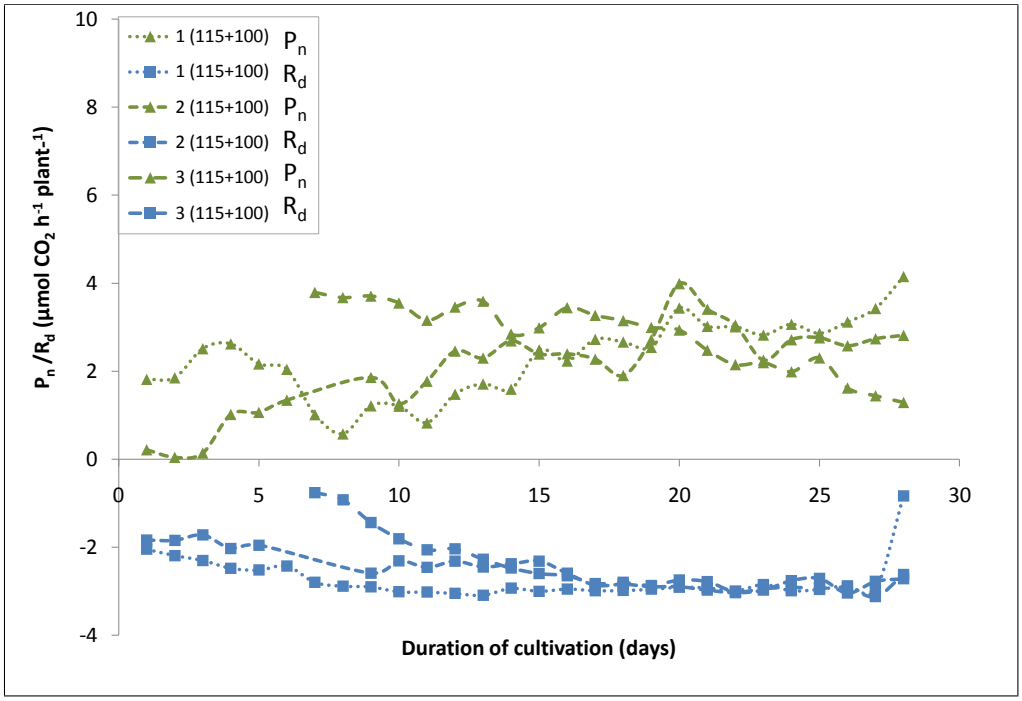
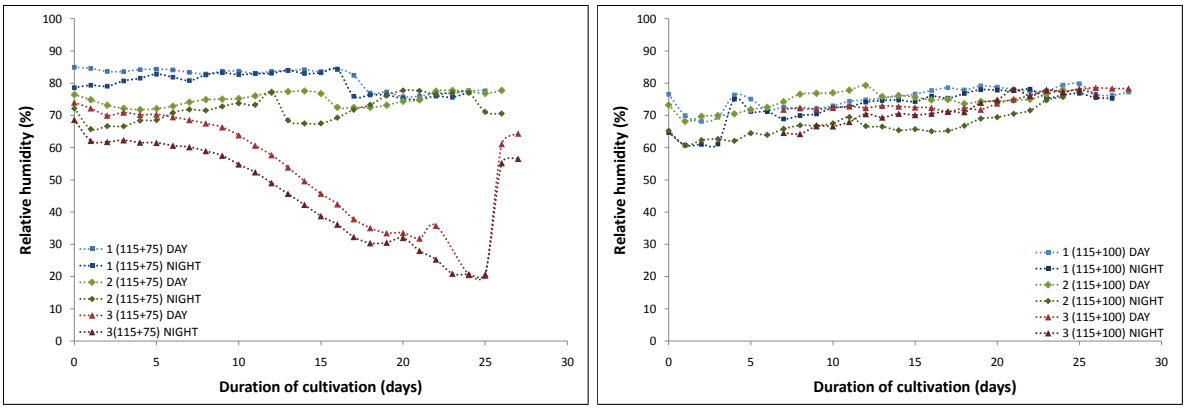


Fig. 5.2: Net photosynthetic rate P_n (day) and dark respiration (night) of the 'Holsteiner Cox' shoots throughout the cultivation in PA-TIS under medium PPFD (115 + 100 µmol m^{-2} s^{-1}), 3 runs.

Increasing the light intensity to 115 + 100 µmol m^{-2} s^{-1} did not result in a higher net photosynthetic rate, but an almost stagnating P_n around 2.4 µmol h^{-1} $plant^{-1}$ (see Fig. 5.2). The maximal P_n was 4.1 µmol h^{-1} $plant^{-1}$, recorded on the last day of the third cultivation. The respiration on the other hand increased slightly over the cultivation, reaching a maximum of -3.1 µmol h^{-1} $plant^{-1}$.

5.1.3 Relative humidity

Due to the high water accessibility within closed cultivation vessels, the relative humidity may rise up to 100 % (Zobayed et al., 2002). In the PA-TIS, rH never surpassed 90 %. In Tab. A.10 (p. 184), all mean values for the treatments under different PPFD are collected.



(a) (b)

Fig. 5.3: HC: Mean relative humidity (rH) in a PA-TIS vessel, divided into day and night, under a PPFD of a) 115 + 75 µmol m^{-2} s^{-1} and b) 115 + 100 µmol m^{-2} s^{-1}. 3 runs.

For all cultivations under a PPFD of 115 + 75 µmol m^{-2} s^{-1}, the relative humidity during the day was higher or equal to the one during the night (Fig. 5.3a). In the third run of this treatment, the rH dropped throughout the cultivation as low as 20 %. This indicates a high loss of humidity.

Under 115 + 100 µmol m^{-2} s^{-1} (Fig. 5.3b), the relative humidity was also higher during the day than during the night. All values lay between 60 and 80 %, with an upward trend in all three repetitions. For the third run, no data was recorded in the first week, because of problems with the data logging.

5.1.4 Temperature

The two different PA-TIS setups (Fig. 4.3 and 4.5, pp. 45 and 47) were located in different rooms. The first tests (under a PPFD of 115 + 75 and 115 + 100 µmol m^{-2} s^{-1}) were run in a laboratory right under the roof, the later ones, presented in Chapter 5.2 (150 and continuous 150 µmol m^{-2} s^{-1}) in a cellar room. As mentioned before, the first cultivation room was not air-conditioned so that the vessels themselves had to be cooled down in order to regulate the temperature. The bottom cooling was used for this purpose (Fig. 4.3, regulation 2).

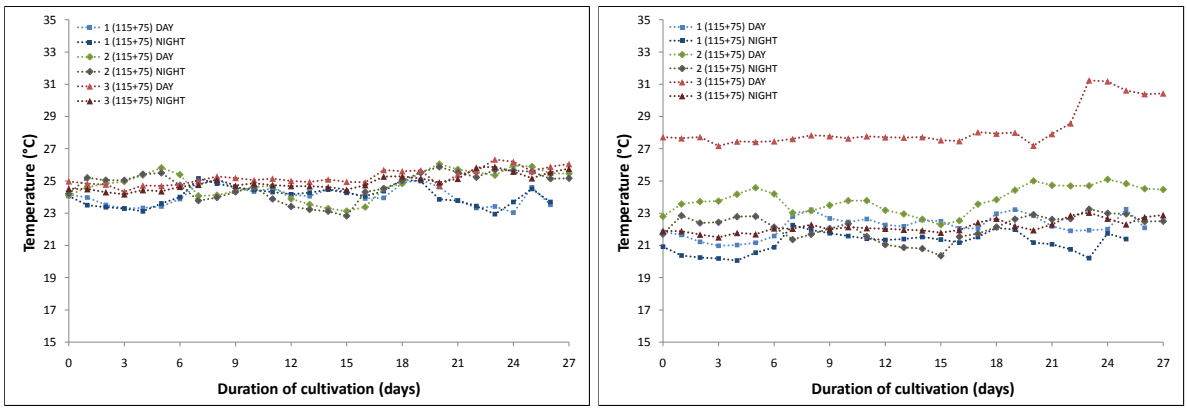
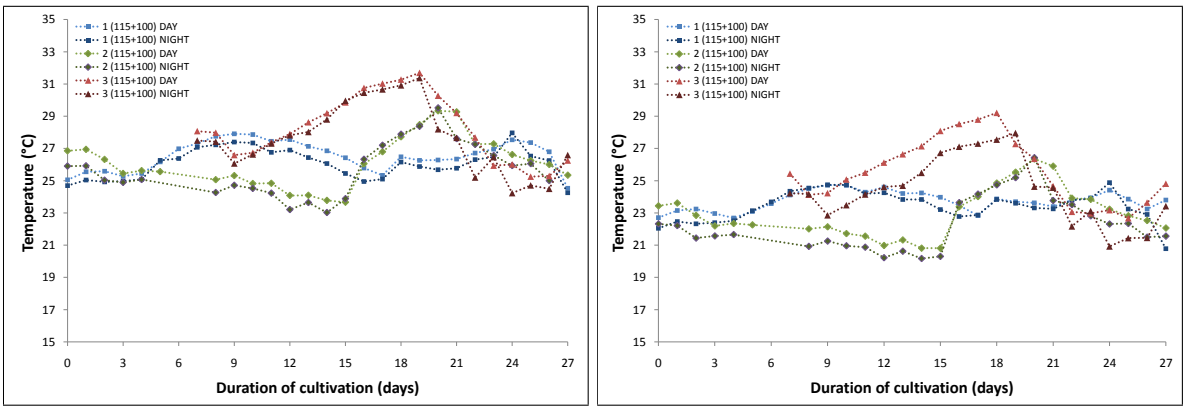


Fig. 5.4: HC: Mean temperature, divided into day and night temperature: a) in a PA-TIS vessel under a PPFD of 115 + 75 µmol m^{-2} s^{-1} and b) room temperature. 3 runs.

For the cultivation of 'Holsteiner Cox' under 115 + 75 µmol m^{-2} s^{-1} (Fig. 5.4a), the day-time mean temperature varied between 24.1 °C and 25.1 °C whereas the night mean temperature lay between 24.1 °C and 24.9 °C (see Tab. A.11, p. 184). The temperature was about 2 K higher within the PA-TIS vessel than outside in the first two runs under these conditions. In the third run, however, the room temperature lay above 27 °C (Fig. 5.4b). This means the bottom cooling succeeded in lowering the vessel temperature to approximately 25 °C. The three runs of this treatment were carried out successively in winter; hence the room was additionally temperated by a radiator. The temperature variation was low under these conditions.

The tests under 115 + 100 µmol m^{-2} s^{-1} took place in spring when the sun heated the flat roof considerably. This heating-up resulted in a room temperature increase, observable in Fig. 5.5b for the second and third run. Cooling the PA-TIS with the bottom cooling was not successful when the room temperature surpassed 27 °C (see Fig. 5.5a). Even in



(a) (b)

Fig. 5.5: HC: Mean temperatures, divided into day and night temperature: a) in a PA-TIS vessel under a PPFD of 115 + 100 µmol m^{-2} s^{-1} and b) room temperature. 3 runs.

the night, the room temperature did not cool down so that the mean temperature was highest in the third run under 115 + 100 µmol m^{-2} s^{-1}. The maximum vessel temperature recorded for all runs was 32.9 °C. The mean temperature and standard deviation of the three runs are therefore much higher than before (Tab. A.11, p. 184). Due to problems with the data logger, the first week of the third cultivation was not recorded.

5.1.5 Plant quality

The 'Holsteiner Cox' shoots in the first setup were cultivated in two different media (MS (+)/(-), including vitamins) under three different conditions (continuous immersion, agar, PA-TIS). Additionally, the intensity of lighting was varied for the plants grown in the PA-TIS. The plants reacted quite differently to the conditions they were submitted to by producing more or less biomass, leaves and roots. Leaf height and root length varied, too. The results for all treatments will be presented in the following.

In the **continuous immersion cultures** (Fig. 5.6a and c) the plant quality was poor. When sugar was present (MS continuous immersion (+)), the plants were hyperhydric and contorted. The leaves were elongated (Fig. 5.8b, p. 74, as compared to MS continuous immersion (-)) and involute. The plants submersed in MS (-) were not hyperhydric, but necrotic and wilted (Fig. 5.6c). The medium they were cultivated in changed color to a reddish-orange (Fig. A.1a, p. 173). Neither treatment lead to a development of roots (Tab. 5.2, p. 75).

The cultivation on **MS agar (+)** (Fig. 5.6b) including vitamins resulted in plants with fully developed leaves and a high number of roots (see Tab. 5.2, p. 75). The roots sprouted from callus and were thin.

Without sugar in the medium, the 'Holsteiner Cox' shoots did not grow as well as with sugar. Less leaves (Fig. 5.8a, p. 74) and roots (Tab. 5.2, p. 75) were produced by the plants grown on **MS agar (-)** (Fig.5.6d).

Plants grown in **PA-TIS** had a heterogeneous quality. Some examples for the deformed shoots detected in PA-TIS are shown in Fig. A.2 (p. 173). Either the plants did not grow at all after the transfer into PA-TIS (a), or the stem was contorted, with roots growing up rather than down (b), or the leaves were glassy and hyperhydric with a thickened stem (c). Especially when lateral lighting was applied, the shoots closest to the light source grew well and produced many roots, like in Fig. 5.6e and f. Further away from the light source, less roots were produced (see Fig. 5.9, p. 76) and the plants were smaller and sometimes hyperhydric. No plants were lost to contamination in PA-TIS.

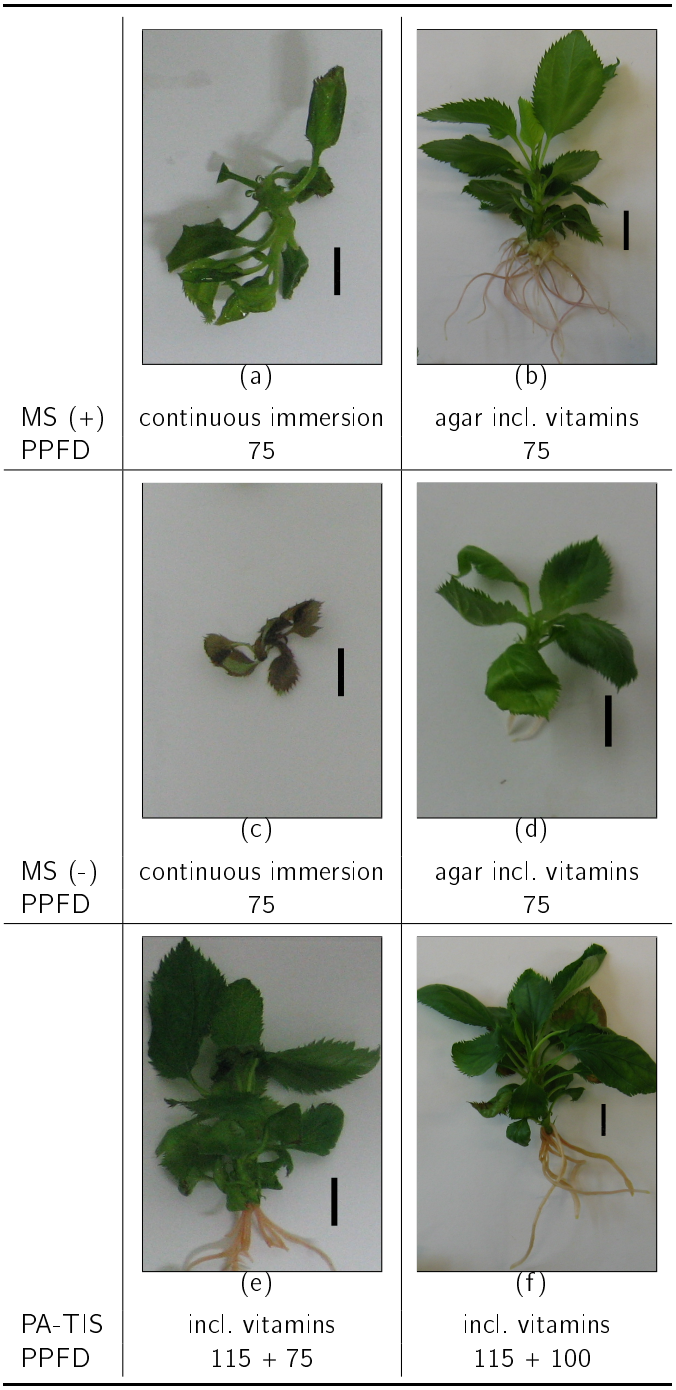


Fig. 5.6: 'Holsteiner Cox' shoots after four weeks of cultivation. Shoots cultivated on MS (+) under a PPFD of 75 µmol m^{-2} s^{-1}, either in continuous immersion (a) or on agar (b). Shoots cultivated on MS (-) under a PPFD of 75 µmol m^{-2} s^{-1}, either in continuous immersion (c) or on agar (d). Shoots cultivated in PA-TIS under varying PPFD : e) 115 + 75 µmol m^{-2} s^{-1} and e) 115 + 100 µmol m^{-2} s^{-1}. Black bar = 1 cm.

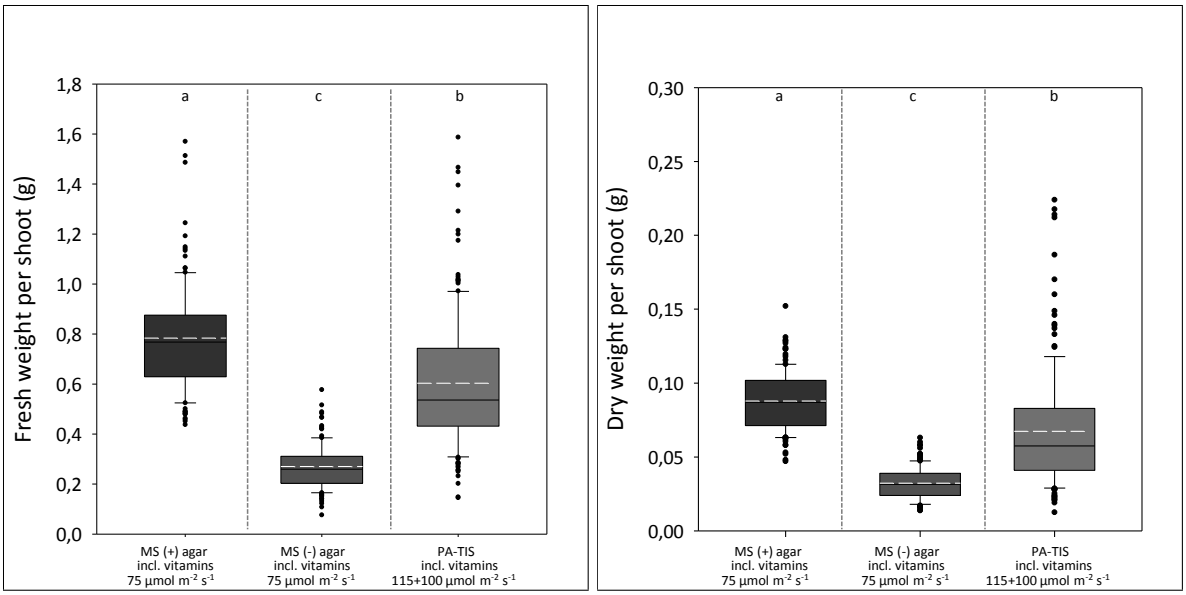
5.1.5.1 Fresh weight, dry weight and dry matter content

The 'Holsteiner Cox' shoots cultivated under different conditions in PA-TIS were always compared to the shoots grown conventionally on MS agar containing sugar or MS agar without sugar. The results of the biomass accumulation (fresh and dry weight) are presented in Tab. A.12, p. 185. Since no statistical differences between the different runs of one treatment were found (Chapter A.1.6), mean values and standard deviations were calculated per single shoot in a treatment. In the first tests (PA-TIS under 115 + 75 µmol m^{-2} s^{-1}), the shoots were not weighed separately, but all plants shoots of one repetition were weighed together. Therefore, no boxplots could be generated. For the later tests, every single shoot was weighed and treated as a repetition for the calculation of mean value and standard deviation. Statistic analyses were conducted for fresh weight, dry weight and dry matter content and are presented in Chapter A.1.6 (p. 187). Since no statistical differences were detected between the three consecutive runs, the values were pooled.

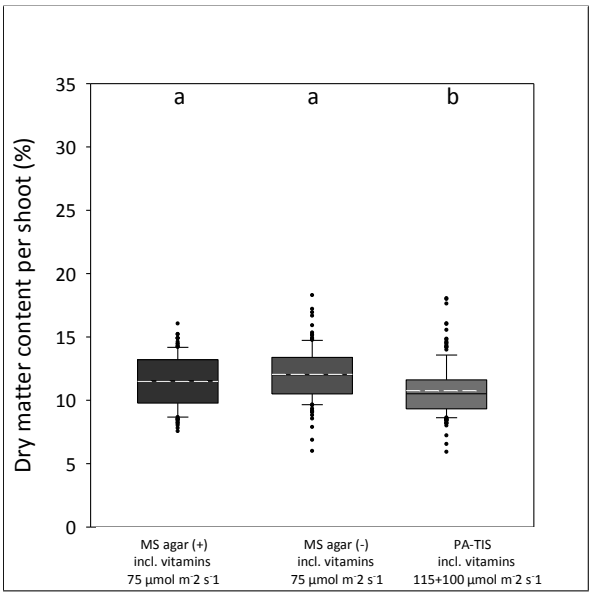
In the first setup, the 'Holsteiner Cox' shoots grown in a MS (+) continuous immersion culture accumulated most fresh weight, but also had the lowest dry weight content (DMC) of all MS (+) treatments due to high hyperhydricity of the material (see Fig. 5.6a and Tab. A.12, p. 185). Unlike the MS (+) continuous immersion culture, the MS (-) continuous immersion culture did not accumulate more biomass than the treatments on MS agar (-). This treatment resulted in the lowest biomass accumulation and DMC of all examined treatments (177 mg and 8.6 %, respectively). On MS agar (+), the shoots accumulated more biomass in the later runs (784 mg as compared to 680 mg).

The plants in the PA-TIS were also cultivated with MS (-) medium, but showed an overall better performance than the plants in continuous immersion or on agar medium. The less successful treatment (under a PPFD of 115 + 75 µmol m^{-2} s^{-1} without vitamins) still accumulated 86 % more fresh weight than the better treatment on MS agar (-) (270 mg vs. 480 mg fresh weight). An increase of PPFD from 115 + 75 to 115 + 100 µmol m^{-2} s^{-1} lead to an increase of fresh weight by 33 %.

The highest dry weight was detected in the MS (+) continuous immersion culture, but the dry matter content (DMC) of the shoots was low due to hyperhydricity (10 %) (see Tab. A.12). For MS agar (+), the highest dry weight was recorded in the later cultivations.



(a) (b)



(c)

Fig. 5.7: (a) Fresh weight, (b) dry weight and (c) dry matter content per 'Holsteiner Cox' shoot after 4 weeks of cultivation on MS agar (+)/(-) incl. vitamins (75 µmol m^{-2} s^{-1}) or in PA-TIS (115 + 100 µmol m^{-2} s^{-1}). Treatments labeled with the same letter do not differ significantly at $p \leq 0.05$ (Dunn's test). AM = white dashed line. 3 runs.

5.1.5.2 Leaves

The 'Holsteiner Cox' shoots were cut to 4 - 5 leaves per explant before the transfer to PA-TIS or the reference cultures in continuous immersion or on MS agar (+)/(-). Statistic analyses were conducted for the leaf length and number. The results are listed in Chapter A.1.6 (p. 187). Since no statistical differences were detected between the three consecutive runs for the treatments, the values were pooled for the boxplots.

In Fig. 5.8a the number of leaves after 4 weeks of cultivation is depicted for all treatments. The highest number of leaves, almost 11 per shoot, was counted for the shoots grown on MS agar (+). As mentioned above, the plants did not react well to the continuous immersion culture in MS continuous immersion (-), so that the lowest number of leaves was detected here ($\tilde{6}$ per shoot). The treatments in MS continuous immersion (+) and MS agar (-) did not differ significantly in the number of leaves and a significantly higher number was produced in PA-TIS under 115 + 75 µmol m^{-2} s^{-1}. The leaf length was on average highest both in PA-TIS and on MS agar (+) (Fig. 5.8b).

In the later tests, when the 'Holsteiner Cox' shoots were cultivated under 115 + 100 µmol m^{-2} s^{-1}, more leaves were produced than under 115 + 75 µmol m^{-2} s^{-1} in PA-TIS (Fig. 5.8a compared to c). Again, significantly lesser and shorter leaves were produced on MS agar (-) than in PA-TIS or on MS agar (+). The highest number of leaves as well as the longest ones were found in PA-TIS (Fig. 5.8d), but the quality was inhomogeneous, so that the mean values for number of leaves and leaf length were still lower than on MS agar (+). A positional effect was detected for the rooting of the shoots and will be presented in the following (Fig. 5.9, p. 76).

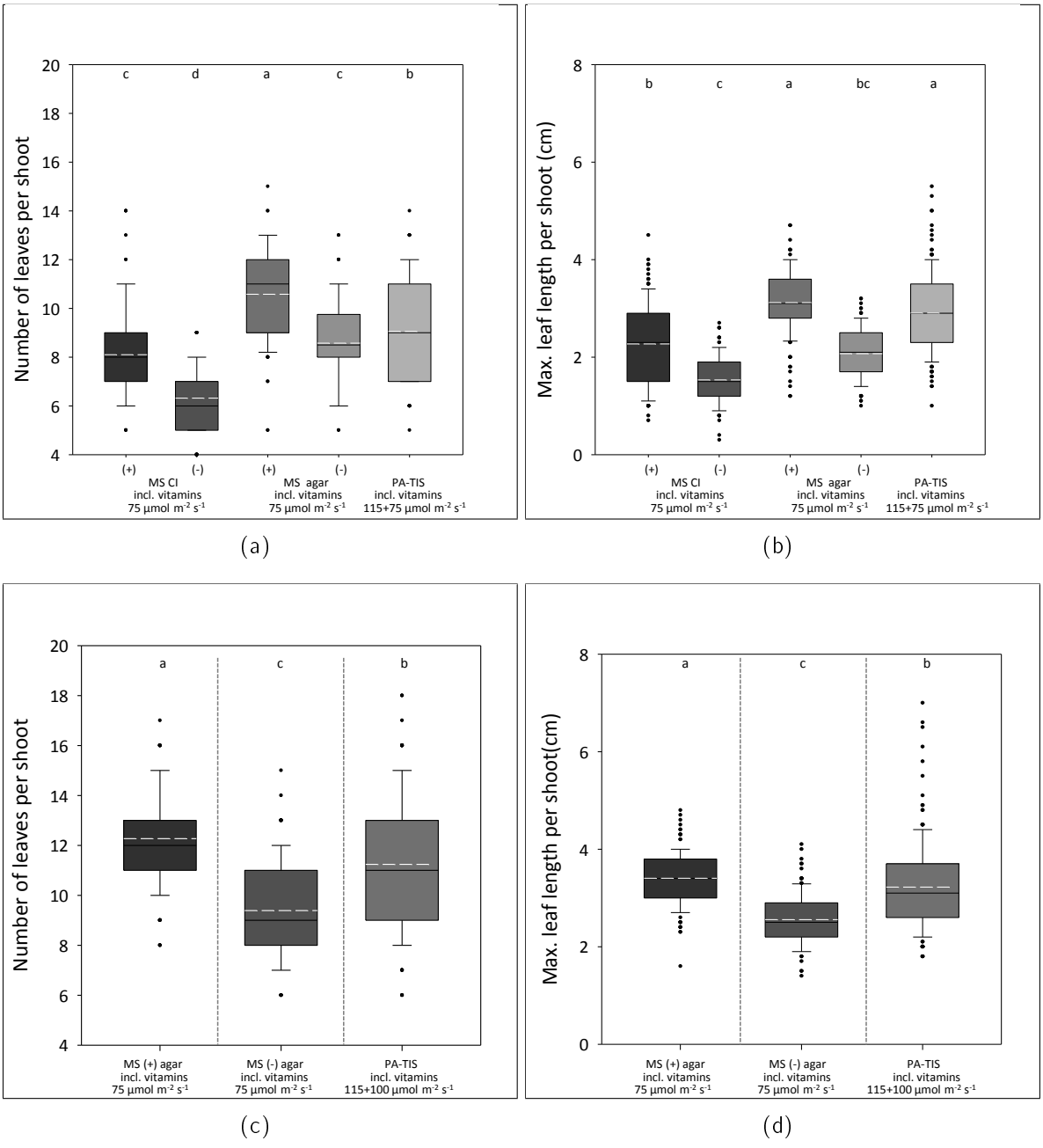


Fig. 5.8: Number of leaves (a) and max. leaf length (b) per 'Holsteiner Cox' shoot after 4 weeks of cultivation on MS agar (+)/(-) incl. vitamins (75 µmol m^{-2} s^{-1}) or in PA-TIS: (a+b): 115 + 75 µmol m^{-2} s^{-1} ; (c+d): 115 + 100 µmol m^{-2} s^{-1}). Treatments labeled with the same letter do not differ significantly at $p \leq 0.05$ (Dunn's test). AM = white dashed line. 3 runs.

5.1.6 Rooting

Tab. 5.2: Rooting success of 'Holsteiner Cox' after 4 weeks of cultivation in the reference cultures and PA-TIS.

Treatment	**Run**	**Vitamins**	**PPFD** ($\frac{\mu mol}{m^2 s}$)	**Rooted shoots /total number**	**Efficiency** (%)
MS Cl (+)	all	+	75	0/120	0
MS Cl (-)	all	+	75	0/120	0
MS agar (+)	1	+	75	40/40	100
MS agar (+)	2	+	75	40/40	100
MS agar (+)	3	+	75	40/40	100
MS agar (-)	1	+	75	5/40	13
MS agar (-)	2	+	75	13/32	41
MS agar (-)	3	+	75	13/40	33
PA-TIS (-)	1	+	115+ 75	40/55	73
PA-TIS (-)	2	+	115+ 75	29/55	53
PA-TIS (-)	3	+	115+ 75	23/42	55
MS agar (+)	1	+	75	40/40	100
MS agar (+)	2	+	75	40/40	100
MS agar (+)	3	+	75	40/40	100
MS agar (-)	1	+	75	7/40	18
MS agar (-)	2	+	75	9/40	23
MS agar (-)	3	+	75	11/40	28
PA-TIS (-)	1	+	115 + 100	39/55	71
PA-TIS (-)	2	+	115 + 100	48/55	87
PA-TIS (-)	3	+	115 + 100	21/55	38

In both continuous immersion cultures (MS agar (+)/(-)), no roots were formed. Therefore, they were not included in Fig. 5.10a+b. A significant difference was found for the rooting between the three runs in PA-TIS under both lighting conditions (Chapter A.1.6). The treatments were hence shown separately in order to discern influences on the rooting efficiency.

Compared to MS agar (-) and PA-TIS, plants cultivated on MS agar (+) including vitamins produced the highest number of roots, which were also the longest recorded on average (Tab. 5.2). In the later cultivation, when the room temperature was higher (see Fig. 5.5, p. 68 and Fig. 5.10c+d), more and longer roots were grown than in the first three runs (Fig. 5.10a+b). In the third run in Fig. 5.10d, however, the root length was significantly lower (3.1 $\pm$ 1.0 cm).

Without sugar in the medium (MS agar (-)), the 'Holsteiner Cox' shoots grew significantly less and shorter roots (Fig. 5.10). Interestingly, more and longer roots were produced in the first three runs (Fig. 5.10a+b than in the later runs Fig. 5.10c+d), a trend opposite to the one detected on MS agar (+).

The number of roots per rooted shoot produced in PA-TIS under 115 + 75 µmol m^{-2} s^{-1} did only differ significantly from MS agar (-) in the first run, for which also the highest rooting efficiency was stated (72.7 %, Tab. 5.2). All runs differed significantly from MS agar (+) (Fig. 5.10a). For the second run , the roots were significantly longer than on MS agar (-) and comparable to those on MS agar (+) (Fig. 5.10b).

For plants grown in the PA-TIS, the PPFD increase from 115 + 75 µmol m^{-2} s^{-1} to 115 + 100 µmol m^{-2} s^{-1} had a positive effect on rooting. The efficiency was higher as well as the average root number and root length (Tab. 5.2). Here, the roots were higher in number and significantly longer than on MS agar (-), but again MS agar (+) surpassed the PA-TIS treatment (Fig. 5.10c+d). The lateral lighting proved to have an important effect on the rooting efficiency. In Fig. 5.9, the root number of the shoots cultivated in PA-TIS under 115 + 100 µmol m^{-2} s^{-1} are shown according to their position in the vessel. The lateral lighting was closer to the vessel than the overhead lamps, resulting in a higher PPFD from the two sides than from above. Plants closest to the lateral lighting had a much higher rooting efficiency than those further away. This effect was unwanted and solved by changing the lighting to 150 µmol m^{-2} s^{-1} only from above (see Fig. 5.29).

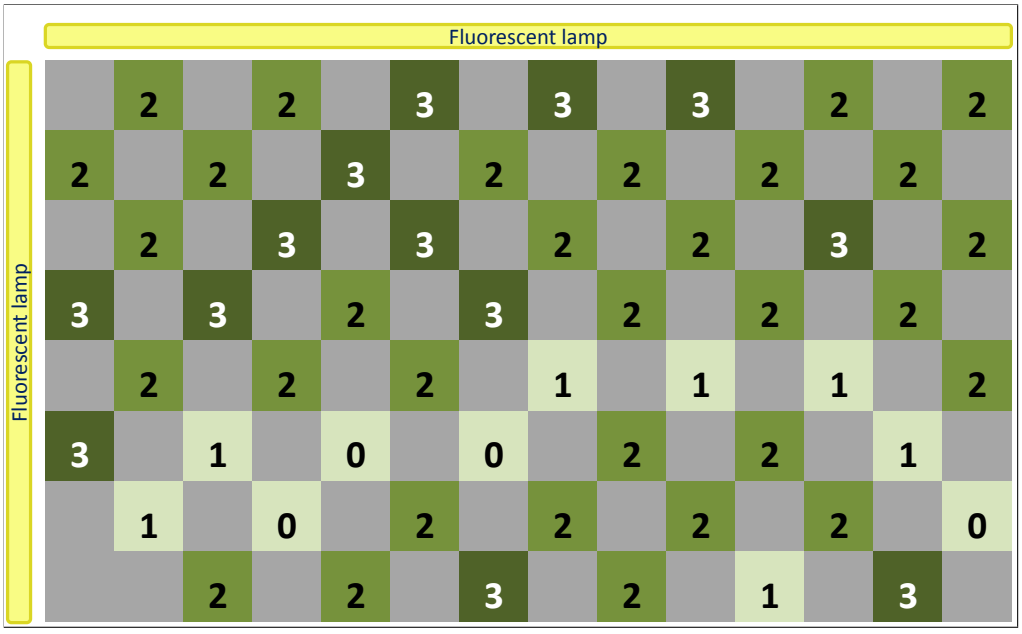


Fig. 5.9: Number of rooted 'Holsteiner Cox' shoots, depending on the position within the PA-TIS vessel (115+100 µmol m^{-2} s^{-1}, lateral lighting shown, 3 runs)

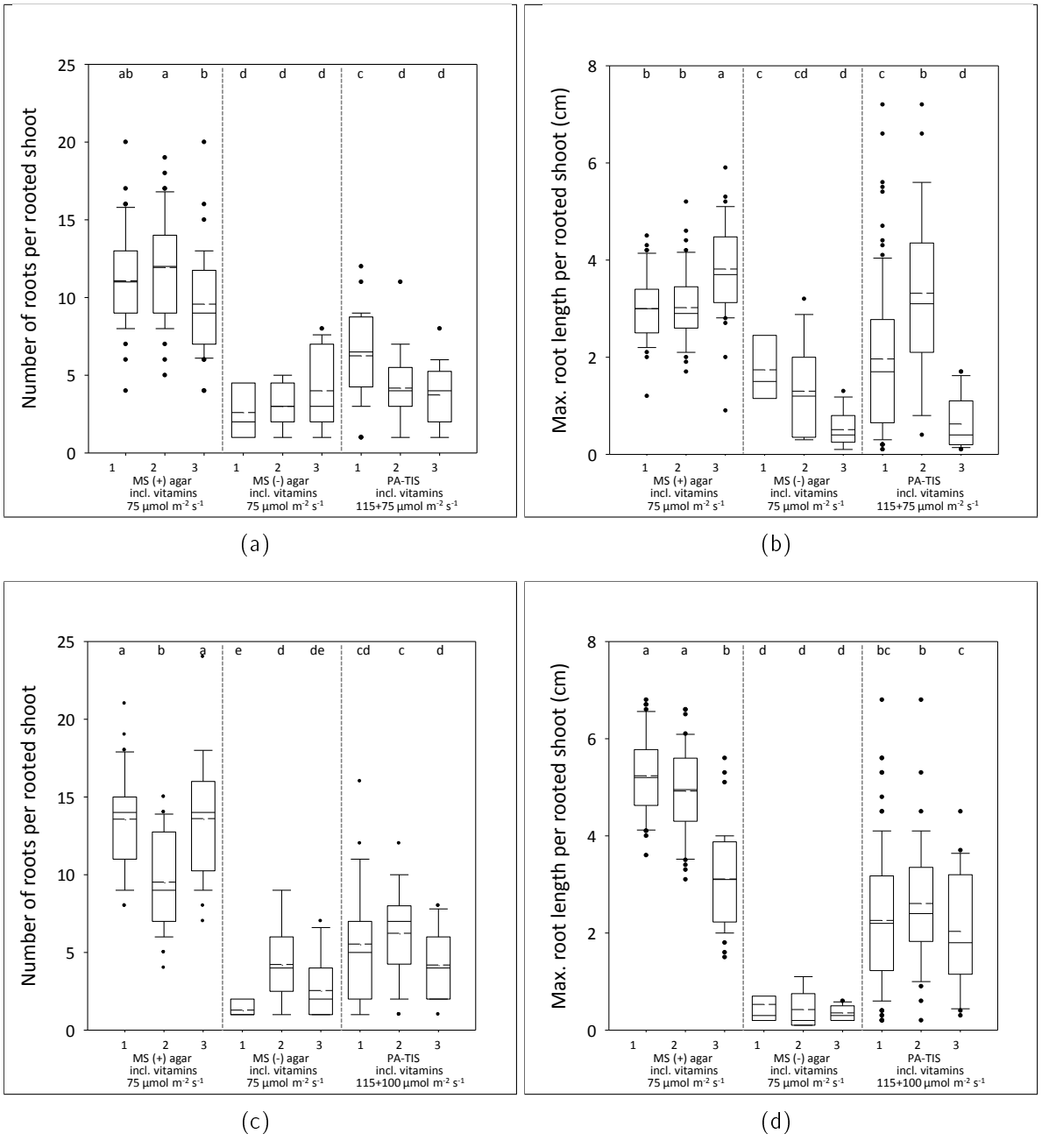


Fig. 5.10: Number of roots (a+c) and max. root length (b+d) per rooted 'Holsteiner Cox' shoot after 4 weeks of cultivation on MS agar (+)/(-) incl. vitamins (75 µmol m^{-2} s^{-1}) or in PA-TIS: (a+b) 115 + 75 µmol m^{-2} s^{-1} or (c+d) 115 + 100 µmol m^{-2} s^{-1}. Treatments labeled with the same letter do not differ significantly at $p \leq 0.05$ (Dunn's test). AM = black dashed line. 3 runs.

5.2 Results of the cultivation of 'Holsteiner Cox' in the second setup (three PA-TIS vessels)

The first setup was changed from a single vessel regulation to the second setup, in which three vessels could be monitored successively. Also, the lighting was changed from lateral plus overhead to solely overhead lighting with a PPFD of 150 µmol m^{-2} s^{-1}. Since the number of vessels was increased, repetitions were possible.

5.2.1 Carbon dioxide and ventilation

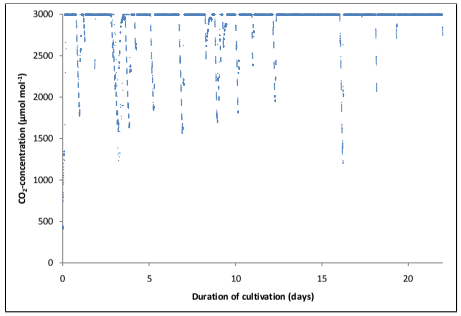
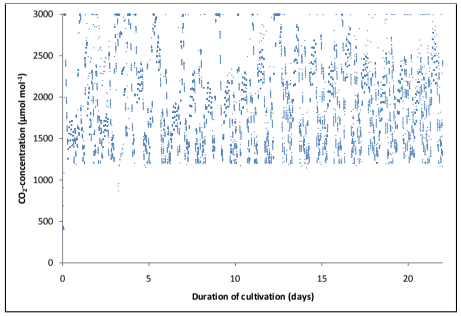
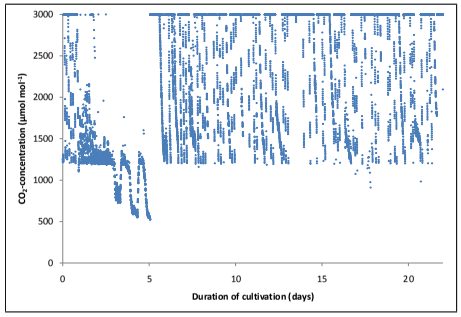
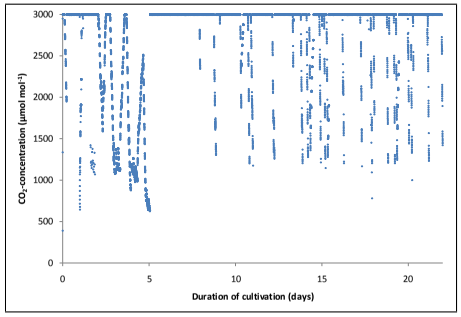
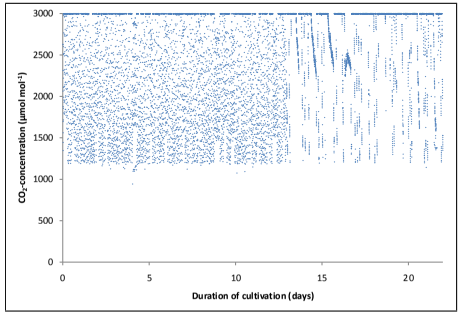
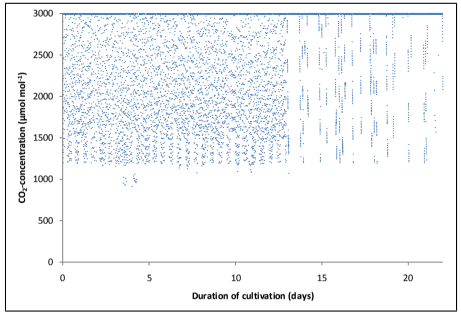
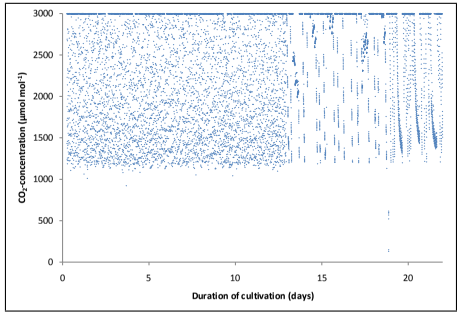
The results of the carbon dioxide regulation are summarized in Tab. 5.3 for the cultivation of 'Holsteiner Cox' shoots under 150 µmol m^{-2} s^{-1} in a 16/8 h light/dark cycle and in Tab. 5.4 for the continuous lighting for 24 h. In the first and second run, only two vessels were used for 'Holsteiner Cox', because the third vessel was used in the cultivation of *Kalmia latifolia*.

The carbon dioxide concentration in vessel 2 during the first run (Tab. 5.3a) under 150 µmol m^{-2} s^{-1} quickly reached the maximum value of 3000 µmol mol^{-1} and rarely dropped below. The estimation of the net photosynthetic rate was hence difficult (see Fig. 5.11).

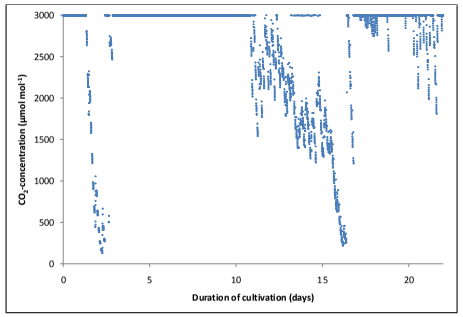
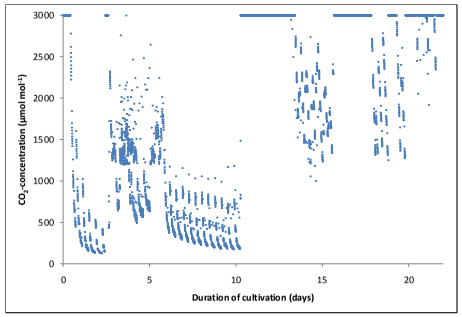
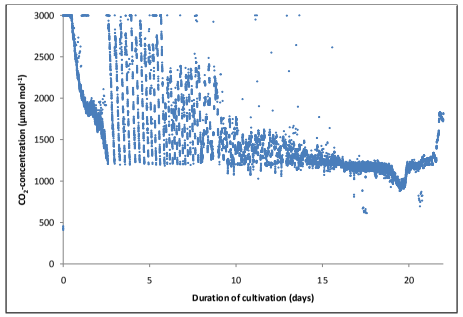
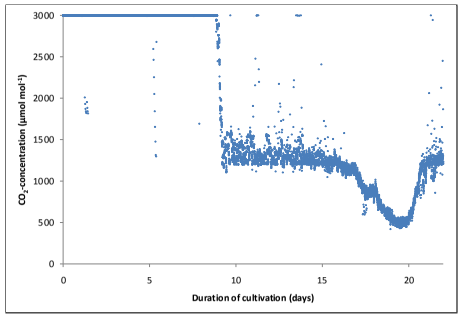
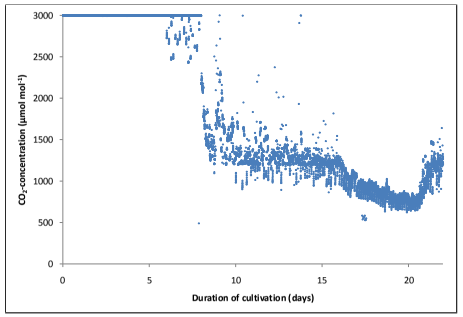
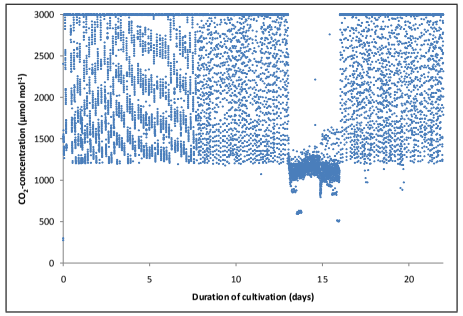
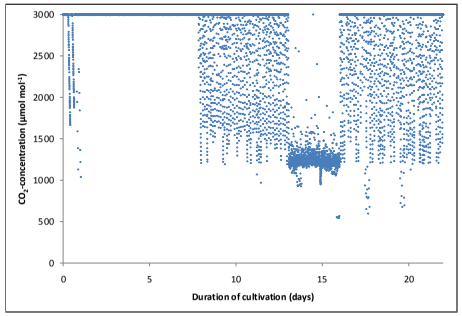
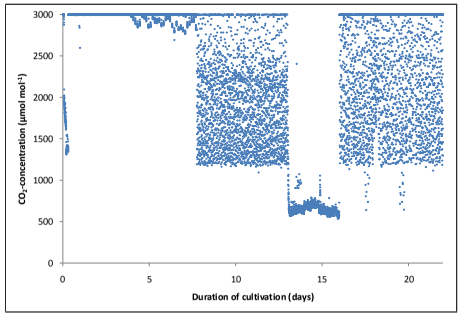
During the first five days of cultivation in the second run under 150 µmol m^{-2} s^{-1} the concentration in the vessel dropped below 1200 µmol mol^{-1} due to an empty CO_2-bottle (Tab. 5.3c+d). As soon as the bottle was replaced, the concentration rose again in both vessels. Again, the carbon dioxide concentration was higher in vessel 2 than in the other repetition. During third run neither an oversupply nor too low concentrations in either of the vessels were detected (Tab. 5.3e-g). Here, the net photosynthetic rate could continuously be estimated (Fig. 5.13).

The concentration under continuous lighting strongly varied throughout the cultivation in all of the vessels. One vessel is not shown, because the temporary immersion was defective and the plants died off (Fig. A.3, p. 174). During the second run, the concentration dropped below 1200 µmol mol^{-1} between day 15 and 20 of cultivation (Tab. 5.4c-e). Similarly, a stagnation around 1200 µmol mol^{-1} was detected from day 13 to 16 during the third run in all three cultivation vessels (Tab. 5.4f-h).

Tab. 5.3: Carbon dioxide concentration within all PA-TIS vessels for 'Holsteiner Cox', 16/8 h light/dark under 150 µmol m^{-2} s^{-1}. **First run**: a) vessel 2; b) vessel 3. **Second run**: c) vessel 1; d) vessel 2. **Third run**: e) vessel 1; f) vessel 2; g) vessel 3.

	Vessel 1	Vessel 2	Vessel 3
First run	Tab. 5.7a	(a) 	(b) 
Second run	(c) 	(d) 	Tab. 5.7b
Third run	(e) 	(f) 	(g) 

Tab. 5.4: Carbon dioxide concentration within all PA-TIS vessels for 'Holsteiner Cox' under continuous 150 µmol m^{-2} s^{-1}. **First run**: a) vessel 1; b) vessel 2. **Second run**: c) vessel 1; d) vessel 2; e) vessel 3. **Third run**: f) vessel 1; g) vessel 2; h) vessel 3.

	Vessel 1	Vessel 2	Vessel 3
First run	(a) 	(b) 	(Not available, pump malfunction)
Second run	(c) 	(d) 	(e) 
Third run	(f) 	(g) 	(h) 

5.2.2 Net photosynthetic rate

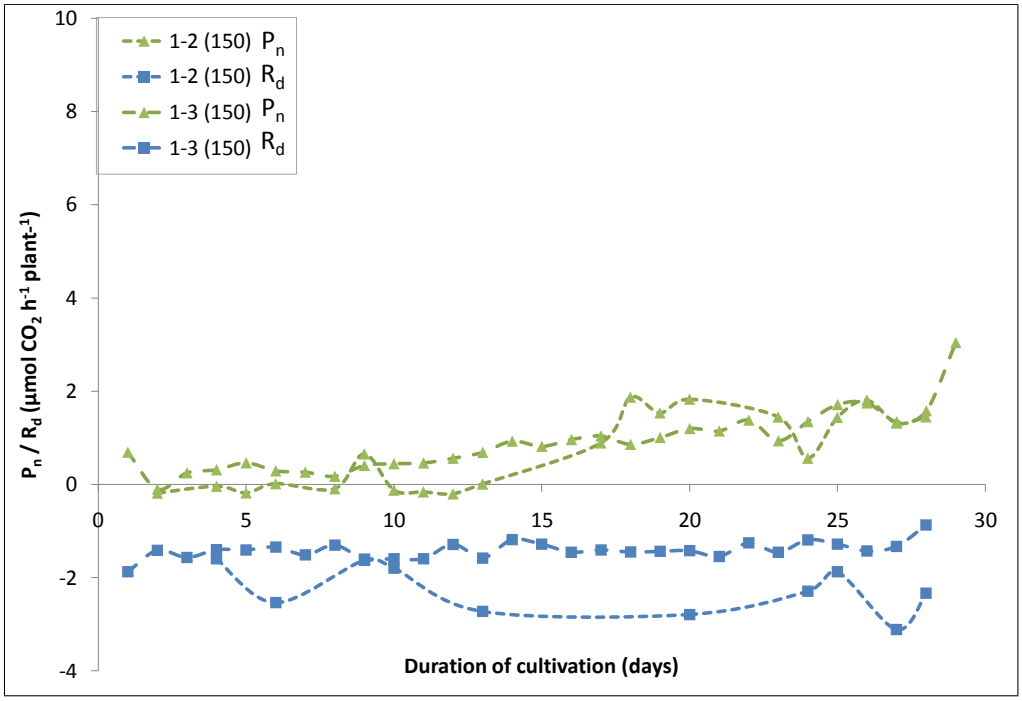


Fig. 5.11: Net photosynthetic rate P_n (day) and dark respiration R_d (night) of the 'Holsteiner Cox' shoots throughout the cultivation in PA-TIS under high PPFD (150 µmol m^{-2} s^{-1}), first run, 2 repetitions.

In Fig. 5.11 to 5.13, the results for the cultivation with a 16 h light and 8 h dark period are shown. In the first two runs, a co-cultivation with *Kalmia latifolia* was conducted in one of the three vessels. Therefore, only two repetitions for 'Holsteiner Cox' were possible.

P_n varied greatly between the different runs, with courses with fairly low P_n in Fig. 5.11, to medium P_n in Fig. 5.12, and a run with high increases of P_n up to 9.4 µmol h^{-1} $plant^{-1}$ (Fig. 5.13). A high variation was also detected for the respiration (R_d), but most values lay between 0 and -2.0 µmol h^{-1} $plant^{-1}$. In the first run (Fig. 5.11), P_n was very low and did not surpass 3.0 µmol h^{-1} $plant^{-1}$. Some values are missing for the cultivation in the second vessel, because the carbon dioxide concentration in the vessel was too high (Tab. 5.3a, p. 79).

The second run shows a course similar to Fig. 5.2, with an almost stagnating P_n above 4.0 µmol h^{-1} $plant^{-1}$ from the sixth day of cultivation to the end. The low P_n in the first few days resulted from a reduced carbon dioxide concentration in the vessel, because the CO_2-reservoir was empty (see Tab. 5.3c and d, p. 79). Again, some values are missing for R_d in the second vessel due to high carbon dioxide production during the

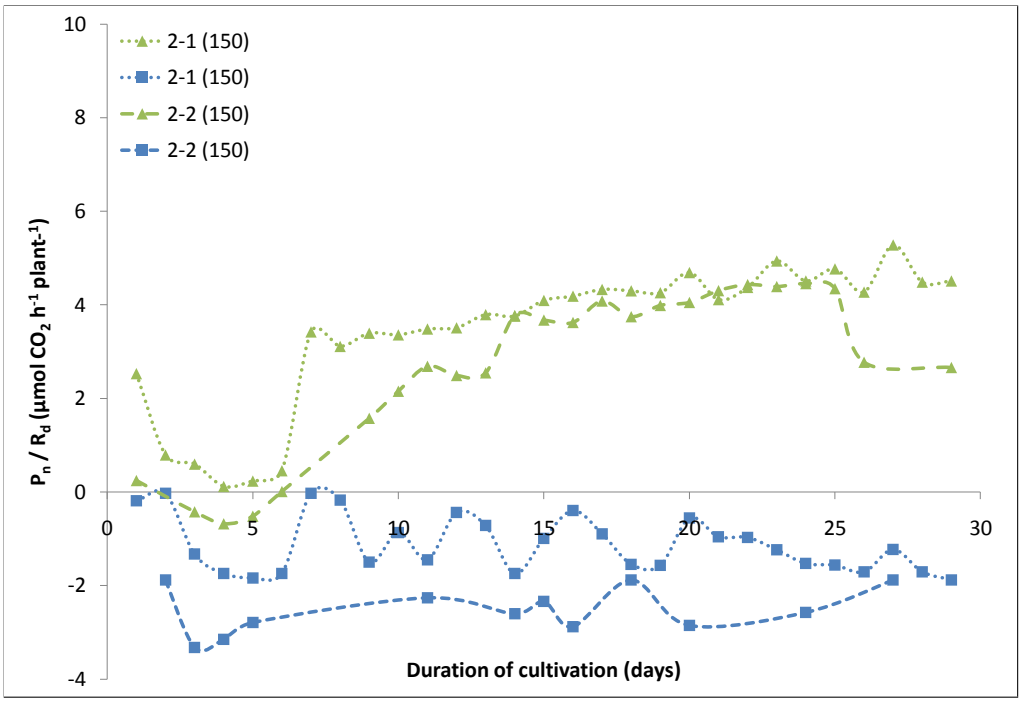


Fig. 5.12: Net photosynthetic rate P_n (day) and dark respiration R_d (night) of the 'Holsteiner Cox' shoots throughout the cultivation in PA-TIS under high PPFD (150 µmol m^{-2} s^{-1}), second run, 2 repetitions.

night (Tab. 5.3d).

Plants grown in the third run (Fig. 5.13) had the highest photosynthetic activity with an increasing P_n of up to 9.4 µmol h^{-1} $plant^{-1}$ throughout the cultivation. R_d averaged -1.0 µmol h^{-1} $plant^{-1}$.

Under full-time lighting, the P_n presented an even greater variability (Fig. 5.14). Photorespiration and photosynthesis both occurred under these conditions, so that the net photosynthetic rate ranged from negative values (respiration) to 5.5 µmol h^{-1} $plant^{-1}$. Most data sets are incomplete, because the carbon dioxide concentration within the vessel rose above the maximum measurable concentration of 3000 µmol mol^{-1} due to respiratory activity and differences could not be calculated anymore.

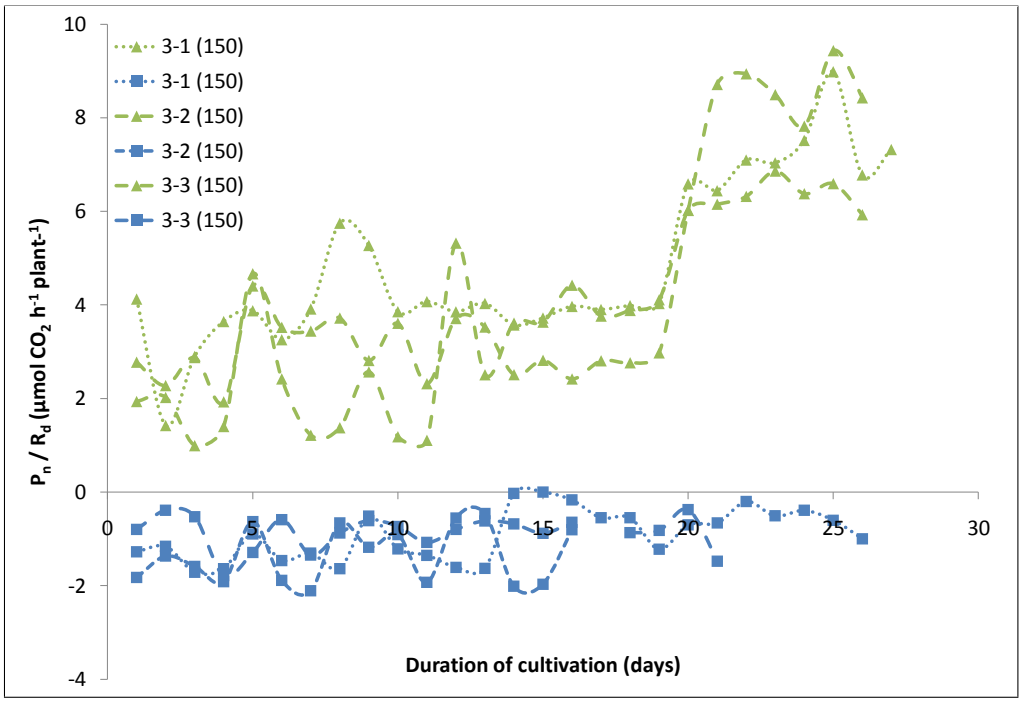


Fig. 5.13: Net photosynthetic rate P_n (day) and dark respiration R_d (night) of the 'Holsteiner Cox' shoots throughout the cultivation in PA-TIS under high PPFD (150 µmol m^{-2} s^{-1}), third run, 3 repetitions.

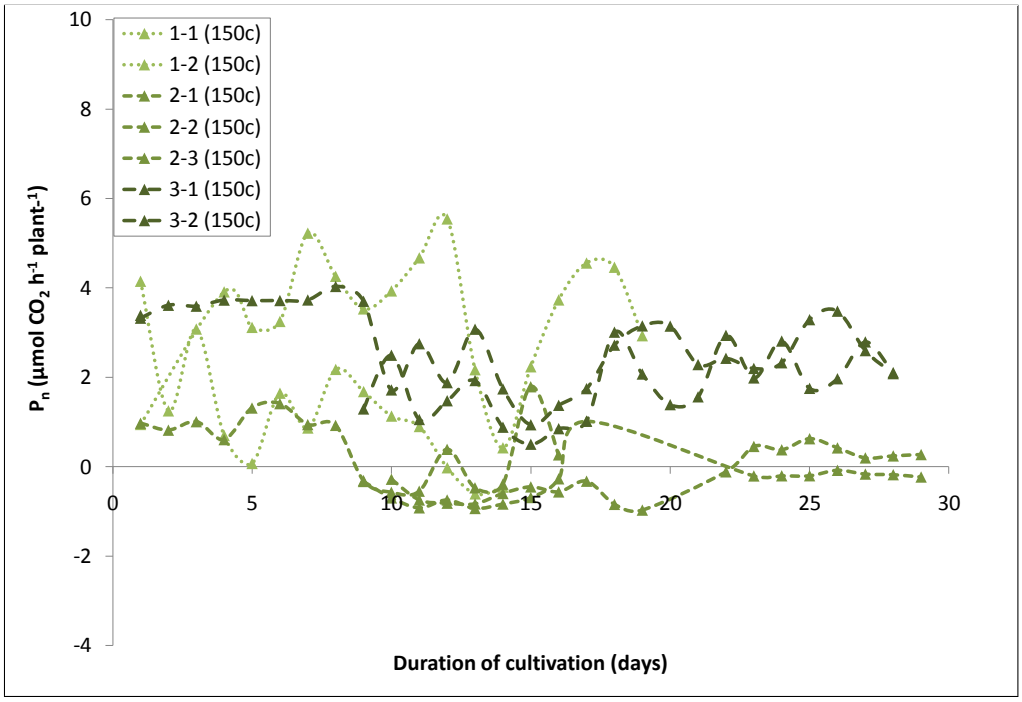
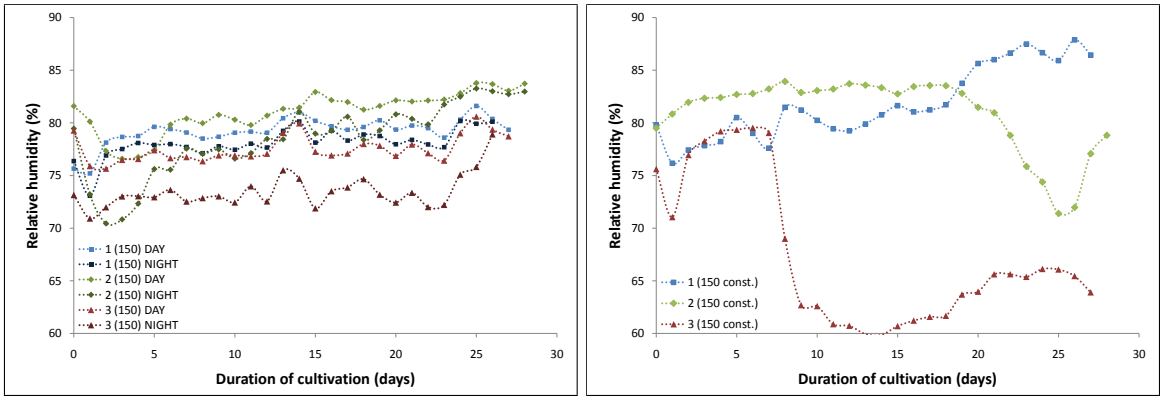


Fig. 5.14: Net photosynthetic rate P_n (day) of the 'Holsteiner Cox' shoots throughout the cultivation in PA-TIS under continuous, high PPFD (150 µmol m^{-2} s^{-1}). 3 runs with 2-3 repetitions.

5.2.3 Relative humidity



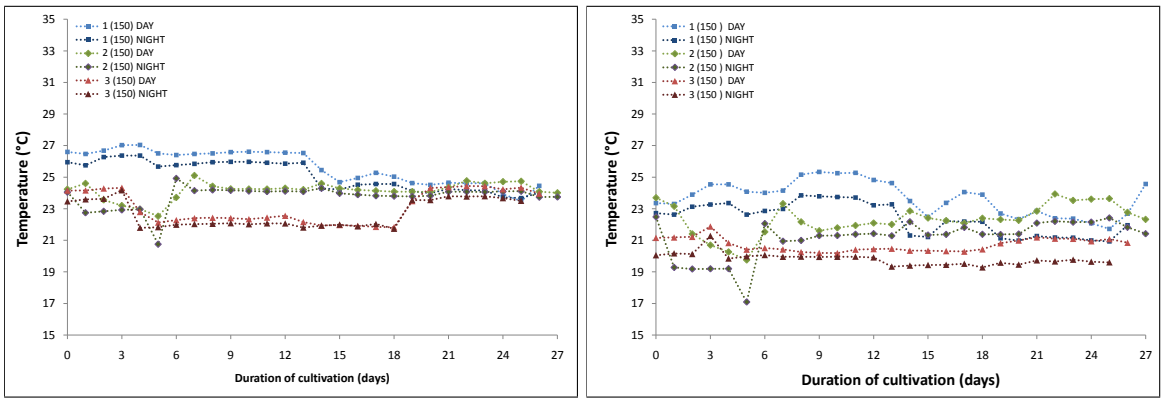
(a) (b)

Fig. 5.15: Mean relative humidity (rH) during the cultivation of 'Holsteiner Cox' in one of three PA-TIS vessels. a) under a PPFD of 150 µmol m^{-2} s^{-1} (16/8 h), divided into day and night; b) under a continuous PPFD of 150 µmol m^{-2} s^{-1} (24 h). 3 runs.

Figure 5.15 shows the results for the cultivation of both 'Holsteiner Cox' lighting treatments in the second setup, in which the vessels were successively monitored for 10 minutes each. Only one of the two or three cultivation vessels simultaneously monitored is presented, since the mean relative humidity did not differ significantly between the single vessels. In Fig. 5.15a, rH did not vary as strongly as in the first setup (Fig. 5.3a or 5.3b), with the lowest humidity at 71 % (night) and the highest at 84 % (day). A slight increase could be stated throughout the course of cultivation. Again, the night-time rH was lower than the day-time. When the plants were continuously lighted rH dropped in the third run from 75-80 % to a value around 65 %.

5.2.4 Temperature

After the room temperature proved to have a significant influence on the vessel temperature (see Fig. 5.5, p. 68), another cultivation room was chosen. The seasonal influence was limited by moving the setup into the cellar and providing an air-conditioning system. The mean temperatures under the new conditions are depicted in Fig. 5.16 and 5.17. During the first run under discontinuous 150 µmol m^{-2} s^{-1} (16/8 h), the room temperature was too high (Fig. 5.16a), so it was lowered in the course of cultivation from 26.5 °C to 25°C. A co-cultivation of 'Holsteiner Cox' and *Kalmia latifolia* in the same room and setup took place; therefore, the room temperature in the third run was further



(a) (b)

Fig. 5.16: HC: Mean temperature, divided into day and night temperature: a) in one of three PA-TIS vessels under a PPFD of 150 µmol m^{-2} s^{-1} and b) room temperature. 3 runs.

reduced.

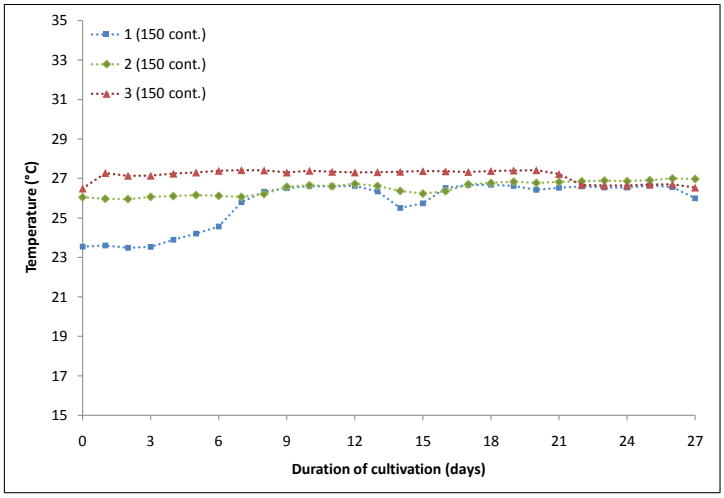


Fig. 5.17: HC: Mean temperature in one of three PA-TIS vessels under continuous 150 µmol m^{-2} s^{-1} in one of three PA-TIS vessels. 3 runs.

When continuous lighting was applied, the mean temperature rose due to the constant heat emission of the lamps. That is why the vessel temperature for these treatments was about 2 K higher on average than for the treatments under discontinuous lighting (Fig 5.17). The room temperature was not recorded under this lighting condition due to problems with the sensor.

The mean temperatures within the cultivation vessels for all cultivations of 'Holsteiner Cox' shoots are collected in Tab. A.11, p. 184.

5.2.5 Plant quality

Tab. 5.5: Summary of all treatments and number of 'Holsteiner Cox' shoots used in the second setup

Treatment	Run	Vitamins	PPFD ($\frac{\mu mol}{m^2 s}$)	N° of shoots
MS agar (+)	1	-	75	40
MS agar (+)	2	-	75	30
MS agar (+)	3	-	75	40
MS agar (-)	1	-	75	40
MS agar (-)	2	-	75	30
MS agar (-)	3	-	75	40
PA-TIS (-)	1	-	150	109
PA-TIS (-)	2	-	150	110
PA-TIS (-)	3	-	150	165
MS agar (+)	1	-	75 (cont.)	20
MS agar (+)	2	-	75 (cont.)	25
MS agar (+)	3	-	75 (cont.)	30
MS agar (-)	1	-	75 (cont.)	25
MS agar (-)	2	-	75 (cont.)	20
MS agar (-)	3	-	75 (cont.)	40
PA-TIS (-)	1	-	150 (cont.)	163
PA-TIS (-)	2	-	150 (cont.)	110
PA-TIS (-)	3	-	150 (cont.)	165

Simultaneously to changing the cultivation room, the setup was changed as well as the medium composition (Fig. 4.5). Instead of one vessel, three vessels were monitored, so that the number of cultivated plantlets was three times higher. Again, the 'Holsteiner Cox' shoots were cultivated on agar medium with or without sugar, but the vitamins were removed from the medium composition. As mentioned before, the quality of the shoots in continuous immersion was very poor, so that only plants grown on agar were used as reference. In the new setup, the duration of lighting was varied for all cultures from 16/8 h day/night to continuous lighting. All treatments are summarized in Tab. 5.5 and the plant quality for each treatment is presented in Fig. 5.18.

The plants grown on **MS agar (+)** without vitamins (Fig. 5.18a) grew less vigorously than those provided with vitamins (Fig. 5.6b, p. 70). The removal of vitamins from the medium resulted in thicker and harder roots that were shorter than before. Less leaves were formed and the shoots were overall smaller. Providing the plants with continuous lighting throughout the cultivation resulted in the poorest plant quality of all MS agar (+) treatments (Fig. 5.18b). More callus was developed and the roots were the shortest (also see Tab. 5.6, p. 99).

Removing vitamins from the medium resulted in smaller plants with few roots for **MS agar (-)** as well (Fig. 5.6c). Unlike plants on MS agar (+), the shoots reacted positively to continuous lighting, producing most roots under this condition as compared to all other cultivations on MS agar (-) (Tab. 5.6). No contamination was detected in any of the vessels without sugar.

Even after changing the lighting direction and intensity to 150 µmol m^{-2} s^{-1} overhead for **PA-TIS**, some plants exhibited a better development than others. A reduction in leaf length as well as number could be stated in general after the removal of vitamins from the medium (Fig. 5.20, p. 92). But for all treatments without vitamins, 'Holsteiner Cox' grew better in PA-TIS than the respective plants on MS agar (+) or (-) (Fig. 5.18e/f compared to a/b or c/d).

Of all the vessels containing MS agar (+) (104 for both setups), 6 were lost to fungal contamination (6 %; see Fig. A.1b, p. 173). Under none of the applied conditions in PA-TIS plants were lost to contamination, but a whole batch (one vessel) withered because a medium pump malfunctioned (Fig. A.3, p. 174). The loss rate was thus 3.8 % (1 of 26 vessels). In the following chapters the results for biomass accumulation (fresh and dry weight) as well as leaf production and rooting efficiency will be presented.

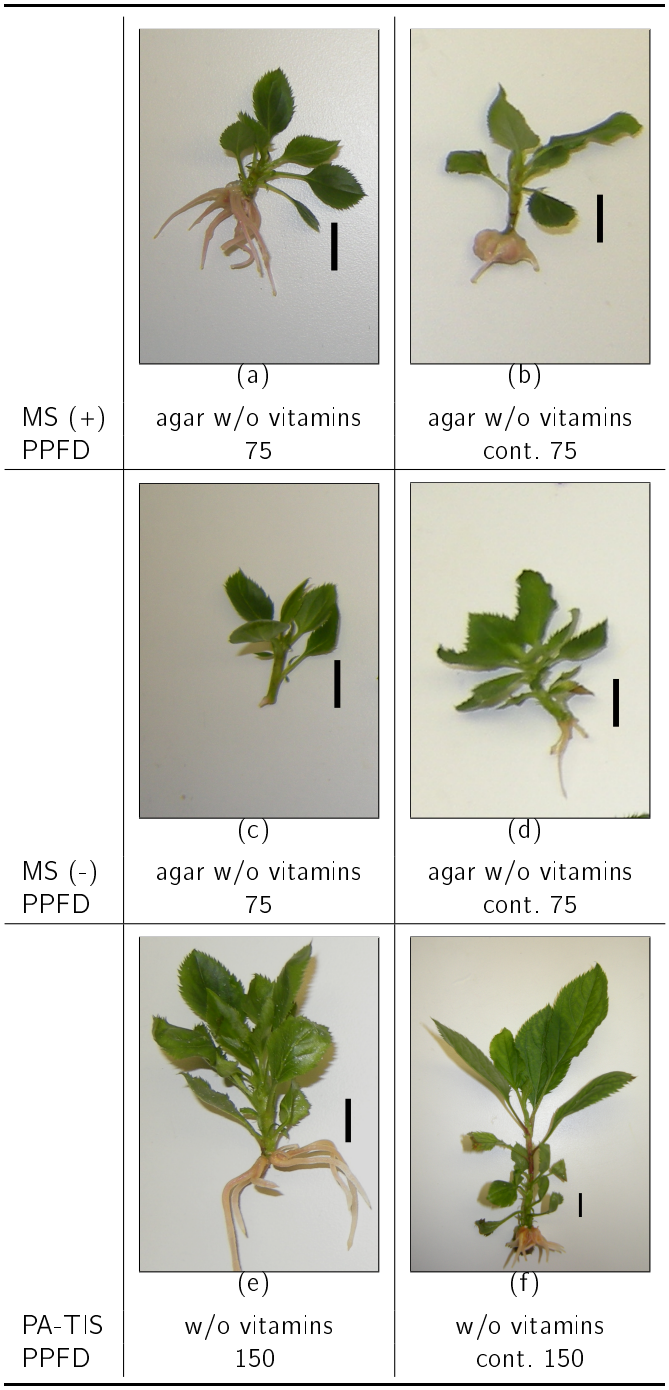


Fig. 5.18: 'Holsteiner Cox' shoots after 28 days of cultivation. Shoots cultivated on MS (+) under a PPFD of 75 µmol m^{-2} s^{-1}, either for 16/8 h day/night (a) or continuous (b). Shoots cultivated on MS (-) under a PPFD of 75 µmol m^{-2} s^{-1}, either for 16/8 h day/night (c) or continuous (24 h) (d). Shoots cultivated in PA-TIS under 150 µmol m^{-2} s^{-1}: 16/8 h day/night (e) or continuous (f). Black bar = 1 cm.

5.2.5.1 Fresh and dry weight

Since all carbon sources should be removed to ensure an actual photoautotrophic growth (Kubota, 2001), no vitamins were added to the media in the present treatments. The results for fresh and dry weight differed significantly between the different runs; hence, they are presented individually in Fig. 5.19.

On MS agar (+), the fresh weight was lower under both lighting conditions without additional vitamins (max. 669 mg) than in the MS agar (+) treatments including vitamins (max. 784 mg) shown in Tab. A.12 (p. 185). The dry weight was lower, too, averaging 50 mg in the best run as compared to 88 mg when vitamins were present. By prolonging the light period from 16 hours to 24 hours continuous lighting, the fresh weight accumulation was increased again (from max. 465 mg to max. 669 mg, compared to 16/8 h lighting). The dry weight increased accordingly and was comparable to the treatments on vitamin-containing medium in Tab. A.12.

For MS agar (-), the same trend as for MS agar (+) was detected after the vitamins were removed from the medium. The fresh weight was reduced from approximately 270 mg to 132 mg and the dry weight from 32 mg to a maximal 19 mg (Tab. A.12). The 24 hour lighting increased the fresh and dry weight again to 218 mg and 28 mg, respectively (first run under continuous lighting, Fig. 5.19b and d). The dry weight of 'Holsteiner Cox' on MS agar (-) was significantly lower than in both other treatments and for lighting conditions, respectively (Fig. 5.19c and d).

The increase of the PPFD to 150 µmol m^{-2} s^{-1} could not compensate for the negative effect of omitting the vitamins which had already been detected in the agar cultures. Less fresh weight was accumulated than in the first setup under lower PPFD (see Tab. A.12, p. 185). Only by increasing the duration of lighting to 24 hours did the production of fresh weight rise again (see Fig. 5.19a compared to b).

Plants grown on MS agar (+) under continuous lighting conditions did not differ significantly for both fresh and dry weight from plants cultivated in the PA-TIS under continuous 150 µmol m^{-2} s^{-1}(see Fig. 5.19b and d).

The dry matter content differed significantly between the different runs for all treatments. For the first run under the new conditions (Fig. 5.19e), the lowest dry matter content was recorded for plants cultivated on both MS agar (+) and (-). A similarly low value was obtained in PA-TIS. Under continuous lighting, no such pattern was found, with a fairly constant DMC in PA-TIS (Fig. 5.19f), and alternating values in the reference cultures.

Compared to the treatments in the first setup, the dry matter content was much higher in the second setup, with mean values as high as 17 % for MS agar (+) and PA-TIS under the 16/8 h light/dark-cycle and equally high values under continuous lighting (Tab.A.12).

5.2.5.2 Leaves

After the vitamins were removed from the medium composition and the 'Holsteiner Cox' shoots were cultivated under 150 µmol m^{-2} s^{-1} and PA-TIS, a significantly higher number of leaves was detected in PA-TIS under 16/8 h light/dark for most runs than in the reference cultures (Fig. 5.20a). The number was, however, much lower than under 115 + 100 µmol m^{-2} s^{-1} as shown in Fig. 5.8c, which corresponds with the results for the fresh and dry weight analysis in the previous chapter. The leaf length, on the other hand, was comparable in both setups (between 2.9 and 3.4 cm).

Under continuous lighting with 150 µmol m^{-2} s^{-1}, the maximum leaf length per shoot was diverse in PA-TIS, with outliers both above and below the mean value of 3.7 cm for all three runs. The longest leaf of all treatments was detected herein (7.5 cm). Significantly shorter and less leaves were produced on MS agar (-) than on MS agar (+) or in PA-TIS. The longer exposure to light resulted in more and longer leaves for both reference cultures and PA-TIS.

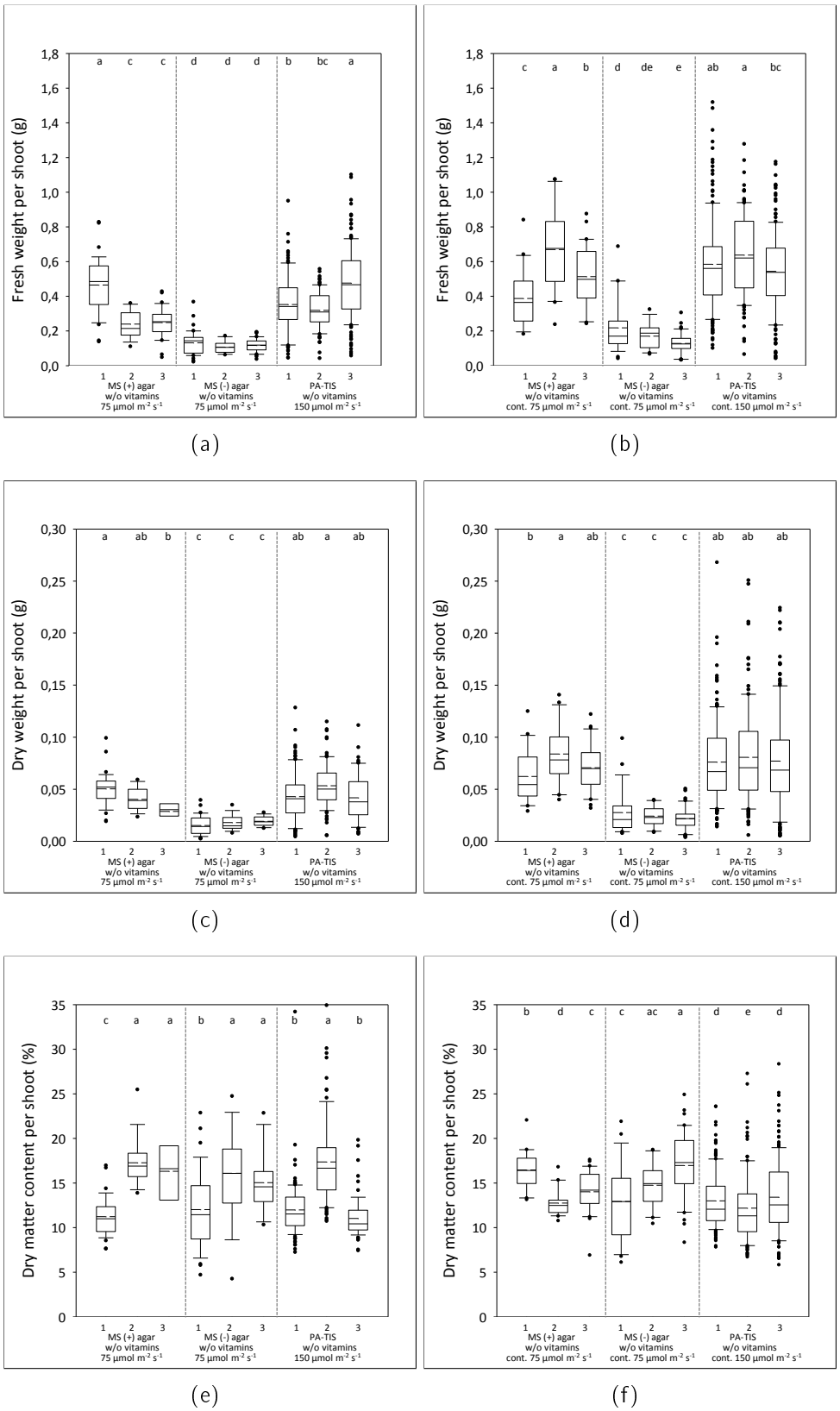


Fig. 5.19: Fresh weight (a+b), dry weight (c+d) and dry matter content (e+f) per 'Holsteiner Cox' shoot after 4 weeks of cultivation on MS agar (+)/(-) w/o vitamins (75 µmol m^{-2} s^{-1}) or in PA-TIS (150 µmol m^{-2} s^{-1}). Lighting: 16/8 h day/night (a, c, e) or continuous (b, d, f). Treatments labeled with the same letter do not differ significantly at $p \leq 0.05$ (Dunn's test). AM = black dashed line. 3 runs.

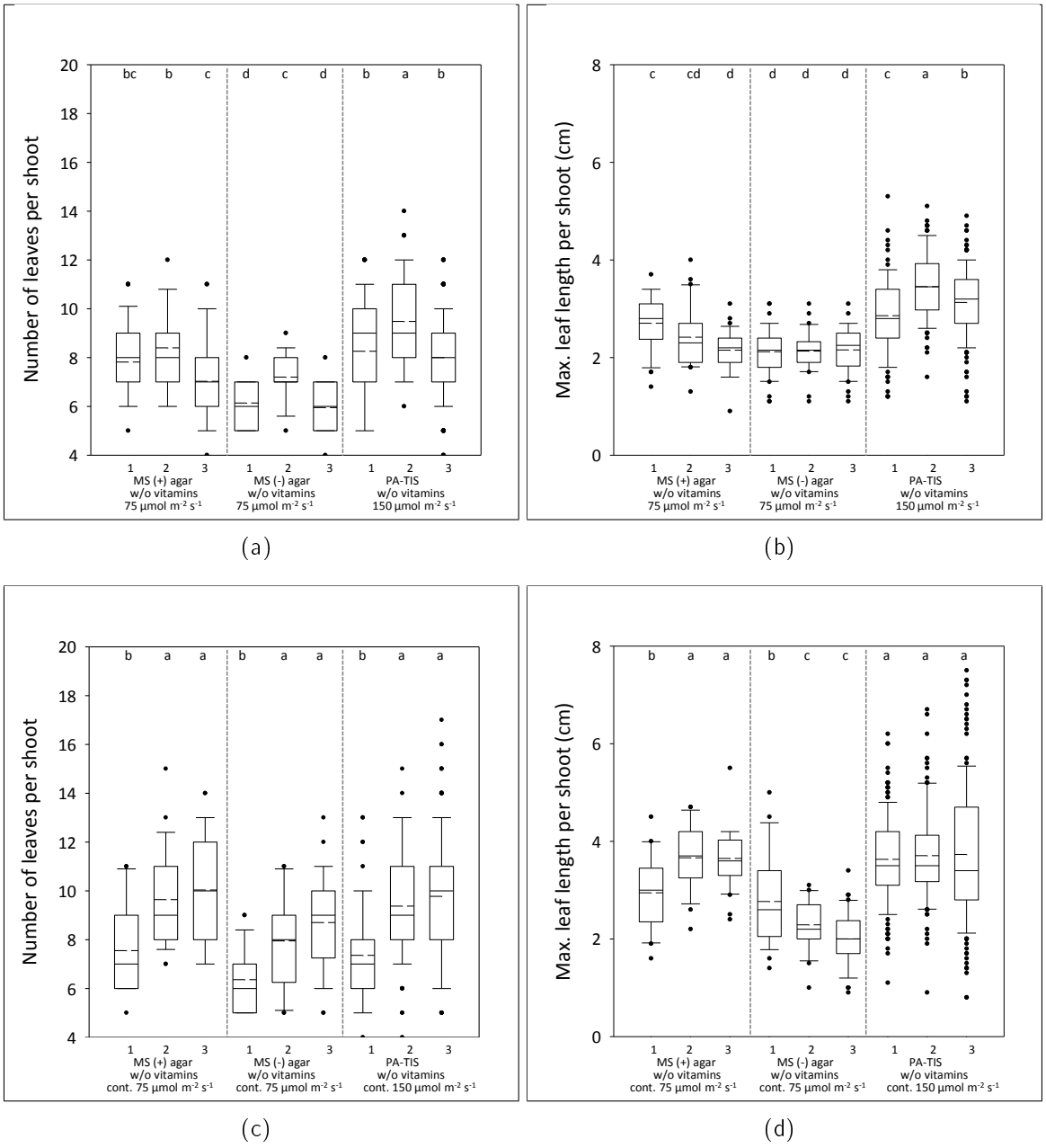


Fig. 5.20: Number of leaves (a+c) and max. leaf length (b+d) per 'Holsteiner Cox' shoot after 4 weeks of cultivation on MS agar (+)/(-) w/o vitamins (75 µmol m^{-2} s^{-1}) or in PA-TIS (150 µmol m^{-2} s^{-1}). Lighting : 16/8 h day/night (a+b) or continuous (c+d). Treatments labeled with the same letter do not differ significantly at $p \leq 0.05$ (Dunn's test). AM = black dashed line. 3 runs.

5.2.5.3 Stomata

The procedure for attaining leaf imprints was described in Chapter 4.3. For this experiment, newly formed leaves of 'Holsteiner Cox' plants grown on MS (+)/(-) agar (without vitamins) under 75 µmol m^{-2} s^{-1} and in PA-TIS (without vitamins) under 150 µmol m^{-2} s^{-1} were used. Shoots adapted to light (8 h) as well as darkness (8 h) were used. All imprints were analyzed for the number of stomata per mm^2 (stomatal density, mm^{-2}), stomata size (mm^2) and stomatal opening (mm^2 opening per mm^2 stomata). The maximum opening was set to 100 %, and closed stomata were set to 0 %. Fig. 5.21 presents all steps in between.

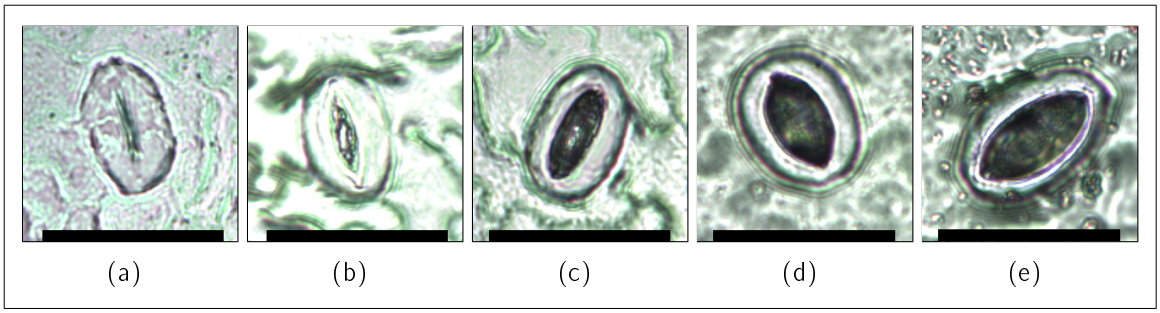


Fig. 5.21: Stomatal opening of 'Holsteiner Cox' ranging from 0 to 100 %: a) closed stoma (0 %), b) slightly opened stoma (- 25 %), c) half-opened stoma (- 50 %), d) mostly opened stoma (- 75 %), e) fully opened stoma (- 100 %). Black bar = 50 μm.

In Fig. 5.25a, the results for stomata size and stomatal density are presented. Shoots grown on MS agar (+) had more stomata per leaf area than plants cultivated either on MS agar (-) or in the PA-TIS. All treatments differed significantly at $p \leq 0.05$. For the stomata size, on the other hand, the opposite trend was detected; the largest stomata were found in the PA-TIS (AM= $8 \cdot 10^{-4}$ mm^2), on average 1.5 times larger than the ones found on MS agar (+) (AM= $5 \cdot 10^{-4}$ mm^2). Again, MS agar (-) lay in the middle (AM= $7 \cdot 10^{-4}$ mm^2).

For all treatments, the stomatal closure was tested under light (a) and after a dark period (b). The results are illustrated in Fig. 5.22 to 5.24.

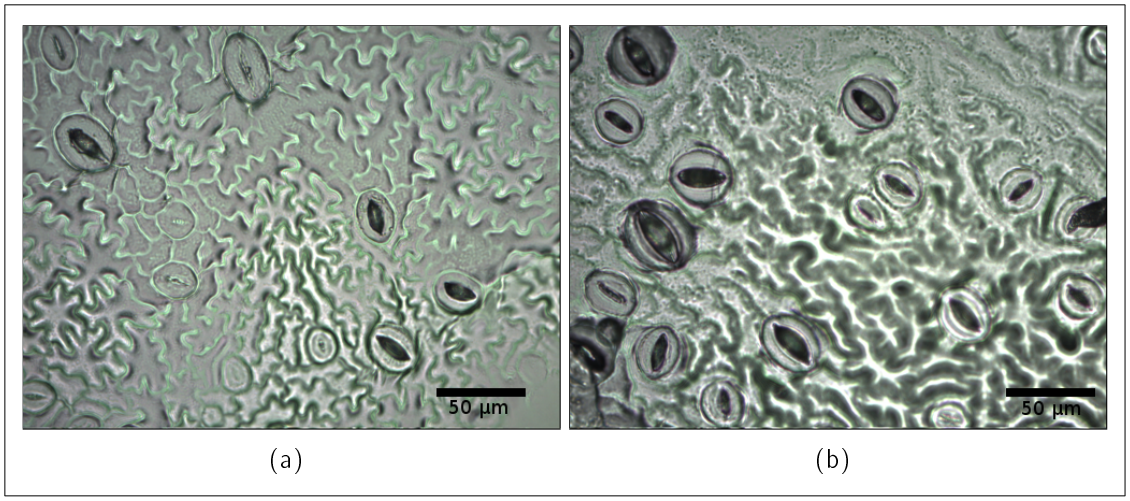


Fig. 5.22: Stomatal opening of 'Holsteiner Cox' on MS agar (+) (a) light, (b) darkness. Black bar = 50 μm

Interestingly, the stomata of plants grown on MS agar (+) were opened wider after a dark period (Fig. 5.22b) than after a period of light (Fig. 5.22a). This trend is also visible when comparing light to dark in Fig. 5.26: on average, the aperture in the darkness (38 %) for plants grown on MS agar (+) was wider than in the light (31 %).

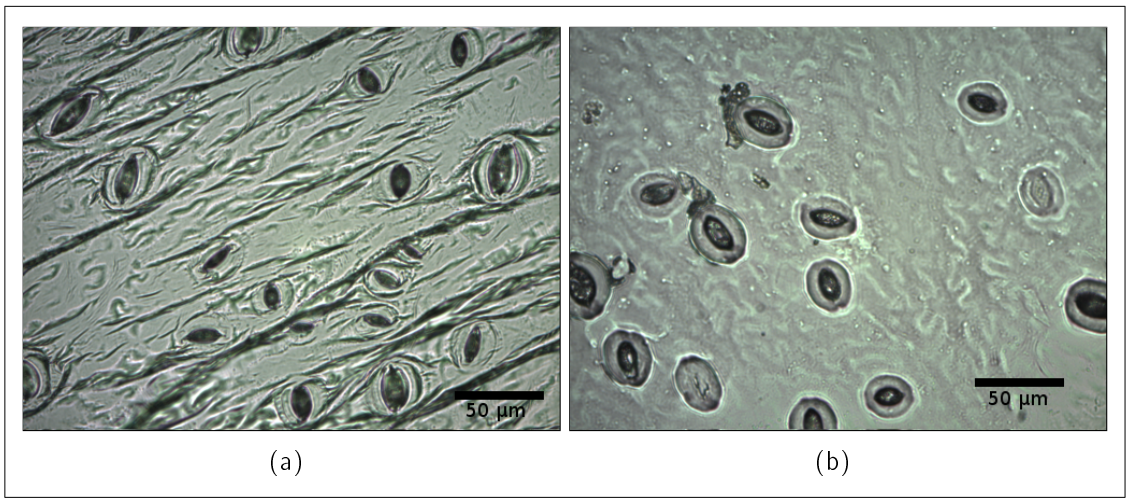


Fig. 5.23: Stomatal opening of 'Holsteiner Cox' on MS agar (-) (a) light, (b) darkness. Black bar = 50 μm

The stomata for 'Holsteiner Cox' grown on MS agar (-) in Fig. 5.23a had a wider opening in the light than the ones on MS agar (+) (Fig. 5.22a). The opening averaged 60 % and was even higher than for shoots grown in the PA-TIS (AM = 50 %, see Fig. 5.26a). In the dark period, the MS agar (-) stomata were not fully closed, but the opening was reduced (Fig. 5.26b).

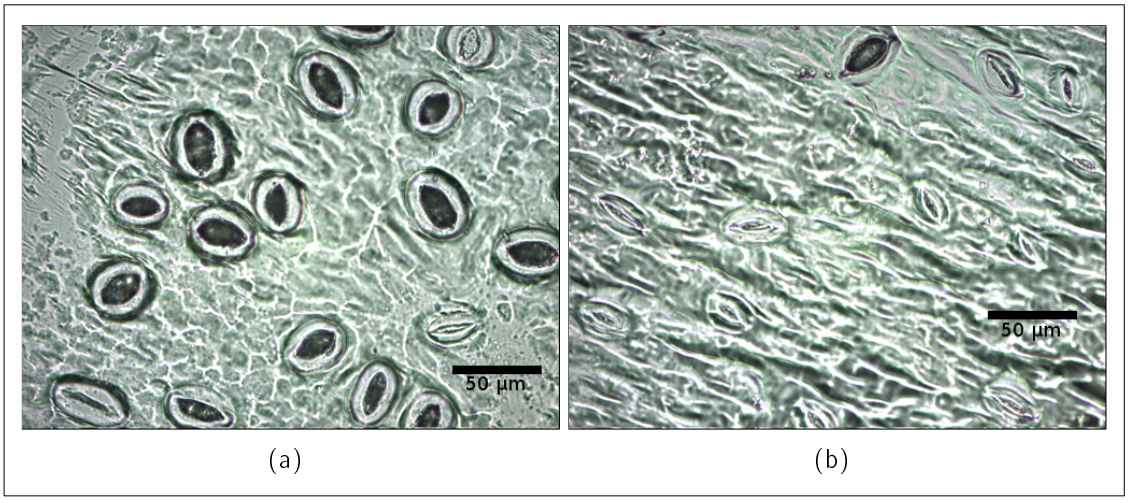


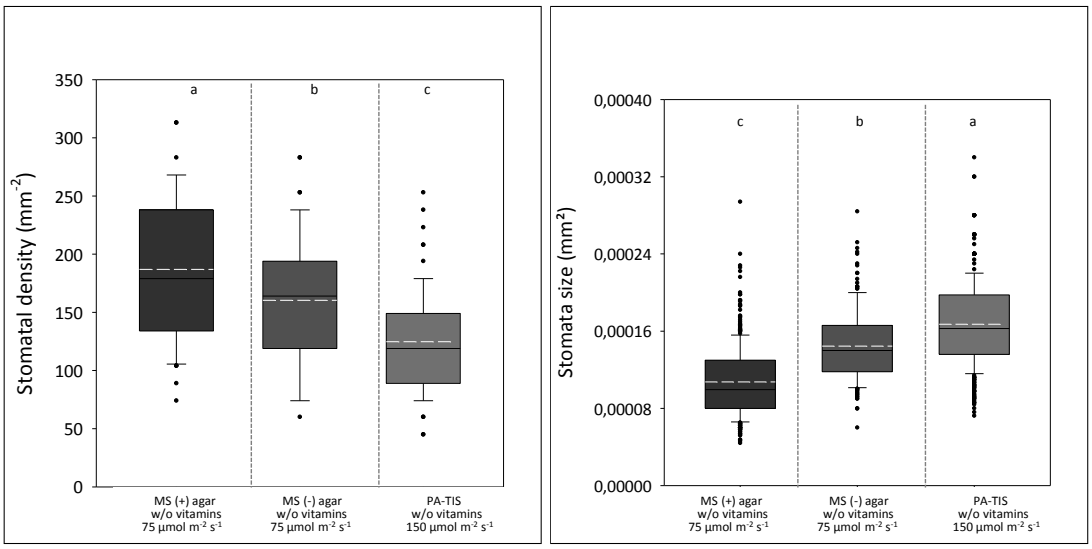
Fig. 5.24: Stomatal opening of 'Holsteiner Cox' in PA-TIS a) light, b) darkness. Black bar = 50 μm

The greatest difference between light and dark was visible for plants cultivated in PA-TIS (Fig. 5.24): under light the opening averaged 50 %, in darkness only 28 % (Fig. 5.26). The stomata were mostly closed, only few were opened more than 50 %.

The opening was tested statistically for all treatments (see labels in Fig. 5.26). For both light and dark period, all treatments differed significantly. In Fig. 5.27, the percentages of stomata divided into the intervals 0, 1 - 25 %, 26 - 50 %, 51 - 75 %, and 76 - 100 % of opening (see Fig. 5.21) are grouped. For MS agar (+), the percentage of stomata opened up to 50 and 75 % increased in the dark period, resulting in a high average opening in the dark (38 %), as already mentioned above. It was also the treatment that had the highest number of fully closed stomata during the light period.

The percentage of stomata opened up to 75 % and 100 % was highest for MS agar (-). Even in the darkness, the stomata opened up to 75 % make up for more than 50 % of all stomata. This is why the highest aperture in the darkness was recorded for MS agar (-) (48 %, see Fig. 5.23b), which was nonetheless a reduction compared to the mean aperture during the light period (60 %).

In the PA-TIS treatment, stomata were not fully closed in the light period, but about 8 % were fully closed in the darkness. Around 80 % of all recorded stomata were not opened more than 50 %, which results in the lowest opening of all treatments with an average 28 % of opening.



(a) (b)

Fig. 5.25: (a) Stomatal density (number of stomata per mm^2 leaf area) on the abaxial surface of 'Holsteiner Cox' leaves; (b) Stomatal size (mm^2); 2-3 replicates from six different leaves. Treatments labeled with the same letter do not differ significantly at $p \leq 0.05$ (Dunn's test). AM = white dashed line.

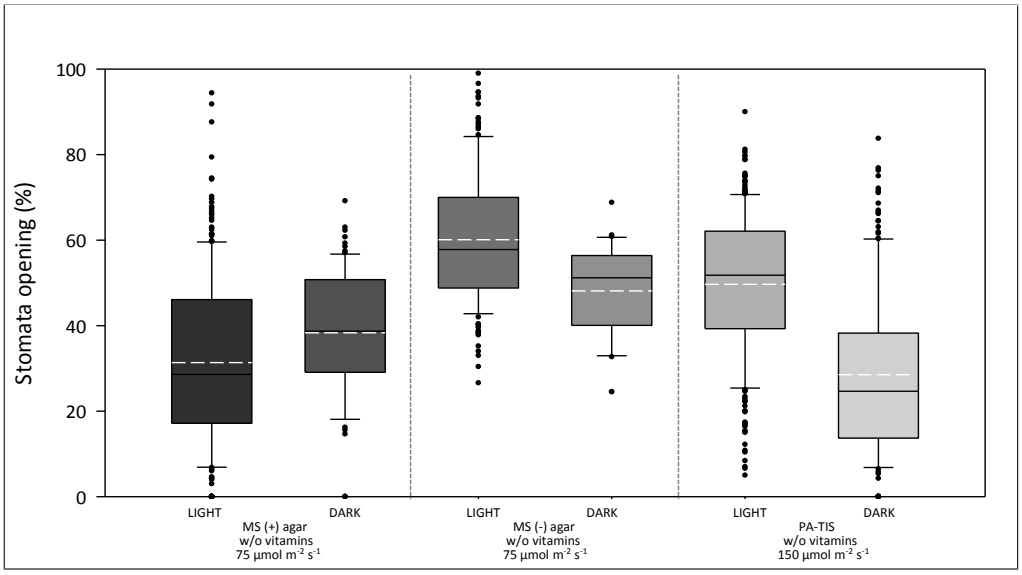


Fig. 5.26: Stomata of the abaxial surface of 'Holsteiner Cox' leaves. Stomatal opening (%) in the light period and dark period for the different treatments; 2-3 replicates from six different leaves. Treatments labeled with the same letter do not differ significantly at $p \leq 0.05$ (Dunn's test). AM = white dashed line.

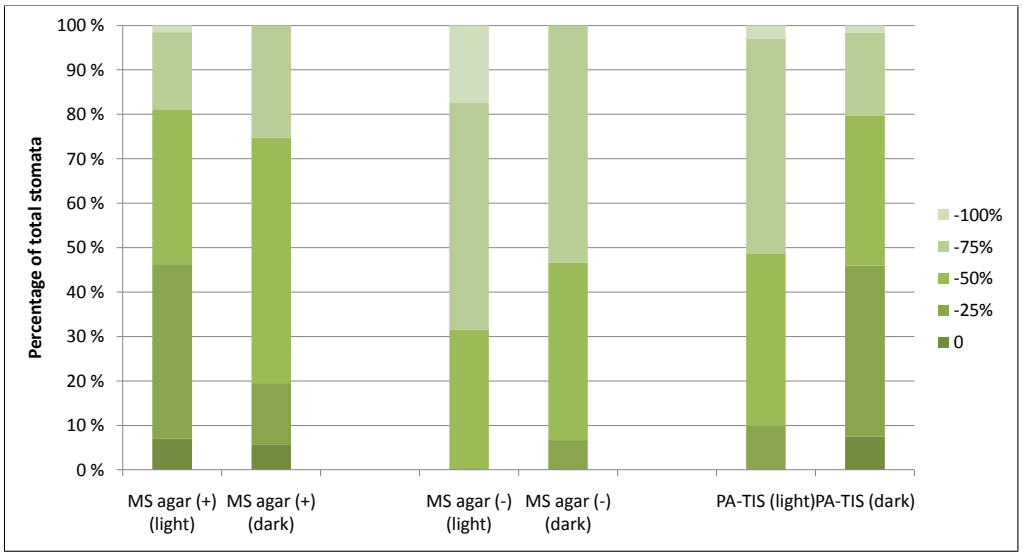


Fig. 5.27: Percentage (%) of stomatal opening (0, -25, -50, -75, -100 %) of all analyzed stomata on the abaxial surface of 'Holsteiner Cox' shoots.

5.2.6 Rooting

The percentage of rooted shoots for the reference cultures and 'Holsteiner Cox' shoots cultivated in PA-TIS under different lighting conditions is summarized in Tab. 5.6. In Fig. 5.28a to d, the root number and length are presented for rooted 'Holsteiner Cox' shoots only. The runs in PA-TIS differed significantly for the root length (Chapter A.1.6), so that they are shown separately.

A negative effect was detected in the reference culture on MS agar (+) when vitamins were removed from the medium composition. The removal resulted in shorter roots than before (see Fig. 5.28b as compared to 5.10b+d, p. 77) and a decrease in the rooting efficiency to as low as 80 % (Tab. 5.6, third run). Continuous lighting did not result in longer roots, but in an increase of the root number and rooting frequency (Tab. A.13, p. 186).

The removal of vitamins from the medium in PA-TIS led to shorter roots, as already observed for plants grown on MS agar (+), even though the PPFD was increased at the same time to 150 µmol m^{-2} s^{-1} (Fig. 5.28b compared to 5.10b+d, p. 77). Under continuous lighting conditions, however, the highest number of roots and rooting efficiency (92 %) in PA-TIS was recorded (Fig. 5.28c). These roots were also the longest for all treatments in PA-TIS (Tab. A.13: 2.8 cm on average in the third run).

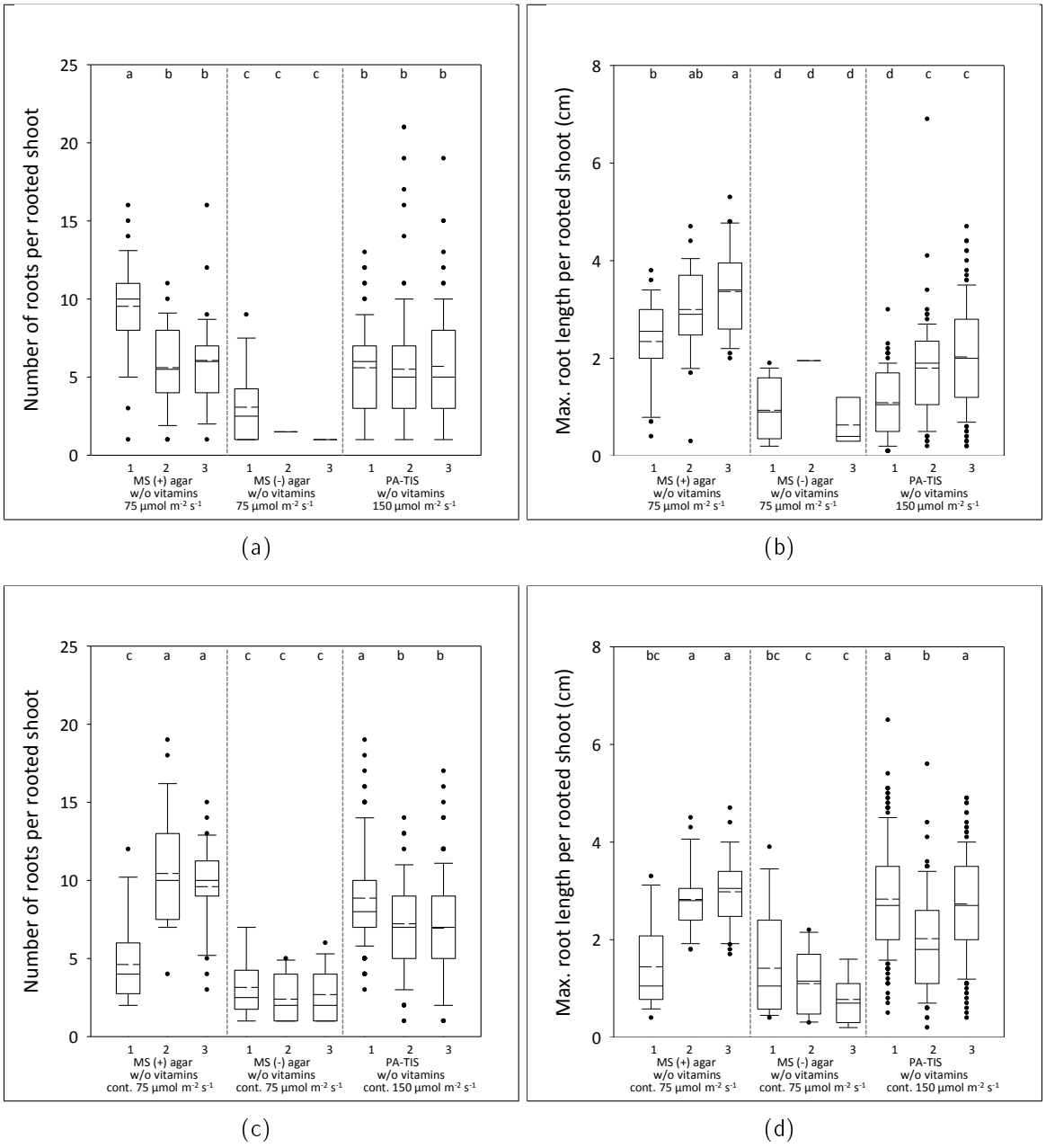


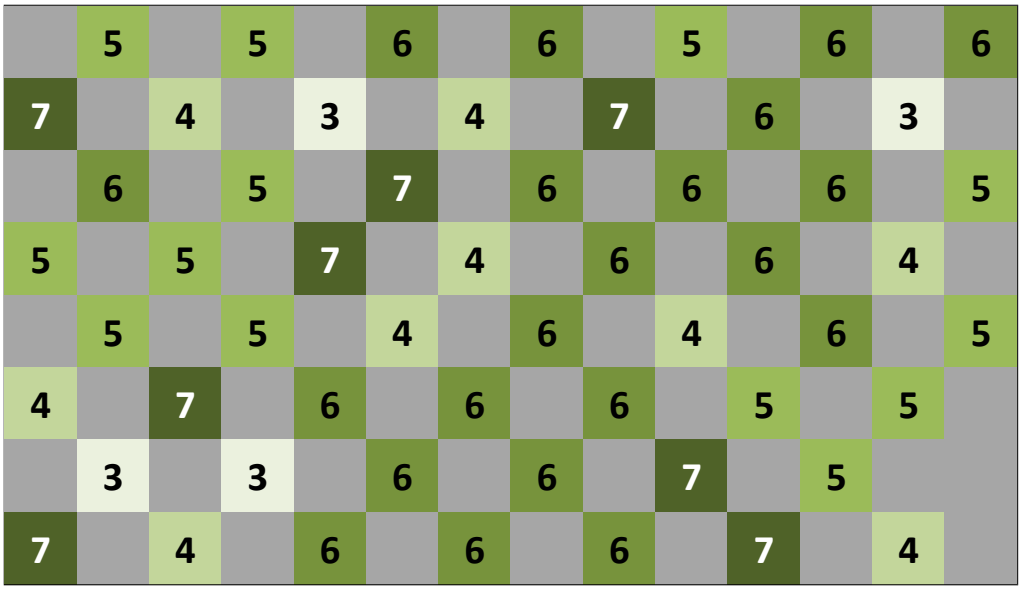
Fig. 5.28: Number of roots (a+c) and max. root length (b+d) per rooted 'Holsteiner Cox' shoot after 4 weeks of cultivation on MS agar (+)/(-) incl. vitamins (75 µmol m^{-2} s^{-1}) or in PA-TIS (150 µmol m^{-2} s^{-1}). Lighting : 16/8 h day/night (a+b) or continuous (c+d). Treatments labeled with the same letter do not differ significantly at $p \leq 0.05$ (Dunn's test). AM = black dashed line. 3 runs.

Tab. 5.6: Rooting success of 'Holsteiner Cox' after 4 weeks of cultivation in the reference cultures and PA-TIS.

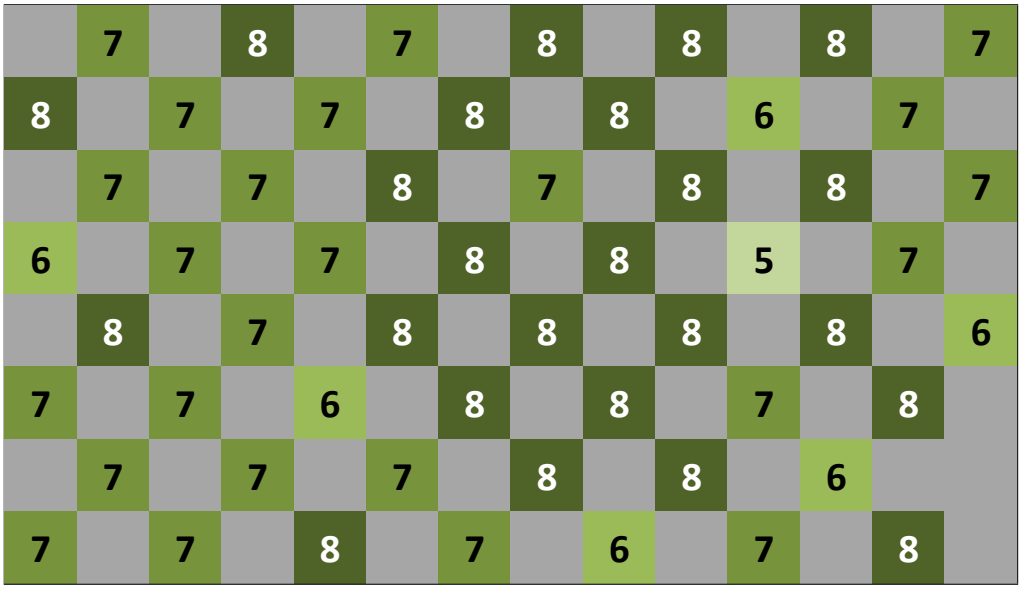
Treatment	**Run**	**Vitamins**	**PPFD** ($\frac{\mu mol}{m^2 s}$)	**Rooted shoots /total number**	**Efficiency** (%)
MS agar (+)	1	-	75	38/40	95
MS agar (+)	2	-	75	28/30	93
MS agar (+)	3	-	75	32/40	80
MS agar (-)	1	-	75	14/40	35
MS agar (-)	2	-	75	2/30	7
MS agar (-)	3	-	75	3/40	8
PA-TIS (-)	1	-	150	84/109	77
PA-TIS (-)	2	-	150	89/110	81
PA-TIS (-)	3	-	150	118/165	72
MS agar (+)	1	-	75 (cont.)	18/20	90
MS agar (+)	2	-	75 (cont.)	25/25	100
MS agar (+)	3	-	75 (cont.)	30/30	100
MS agar (-)	1	-	75 (cont.)	14/25	56
MS agar (-)	2	-	75 (cont.)	10/20	50
MS agar (-)	3	-	75 (cont.)	16/40	40
PA-TIS (-)	1	-	150 (cont.)	157/163	96
PA-TIS (-)	2	-	150 (cont.)	101/110	92
PA-TIS (-)	3	-	150 (cont.)	138/165	84

Without sugar in the medium (MS (-) agar), the 'Holsteiner Cox' shoots grew significantly less and shorter roots (Fig. 5.10 vs. 5.28). The prolongation of lighting to 24 h showed a significant effect for plants on MS agar (+): the rooting efficiency was almost doubled (from 25 to 47 %) under continuous lighting (Tab. 5.6).

For lateral lighting, a positional effect on the growth and rooting of the 'Holsteiner Cox' shoots had been detected (see Fig. 5.9, p. 76). The rooting efficiency under the new conditions in the second setup is depicted in Fig. 5.29a and 5.29b, the latter under continuous lighting. Positional effects were not obvious under both lighting conditions.



(a)



(b)

Fig. 5.29: Number of rooted 'Holsteiner Cox' shoots, depending on the position within the PATIS vessel under 150 µmol m^{-2} s^{-1} for a) 16/8 h light/dark, 7 repetitions; b) 24 h light, 8 repetitions

5.3 Results of the cultivation of Kalmia latifolia

5.3.1 Carbon dioxide and ventilation

During the cultivation of *Kalmia latifolia* in PA-TIS a simultaneous control of 'Holsteiner Cox' and *Kalmia* with the same control system took place during the first two runs (see Tab. 5.3, p. 79). The results are depicted in Tab. 5.7. In the third run, on the other hand, only a single PA-TIS vessel containing *Kalmia* shoots was monitored, so that the density of data is much higher in Tab. 5.7c.

5.3.2 Net photosynthetic rate

The net photosynthetic rate was calculated according to Eq. 4.5 (p. 54). The results are shown in Fig. 5.30.

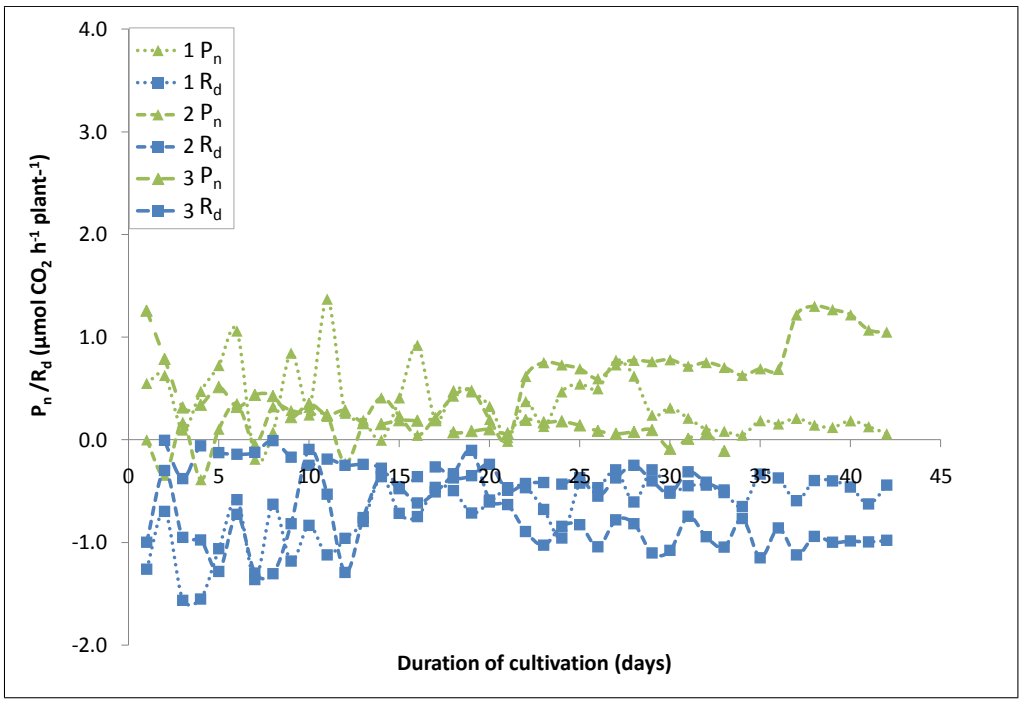
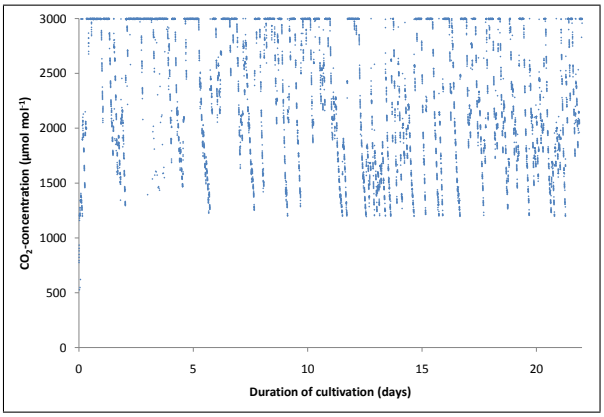
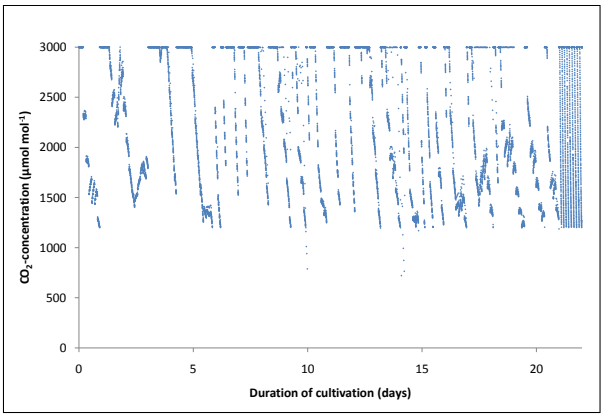
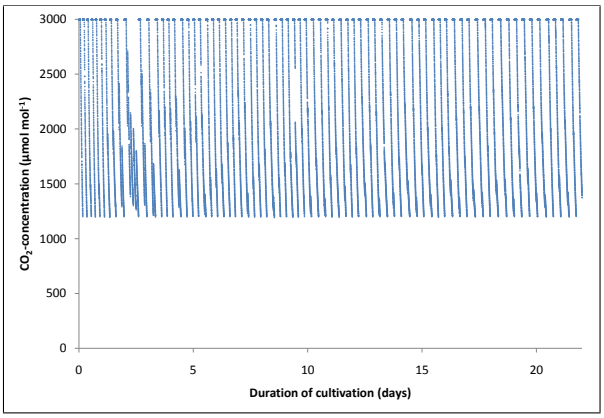


Fig. 5.30: Net photosynthetic rate P_n (day) and respiration (night) of the *Kalmia* shoots throughout the cultivation in PA-TIS under high PPF (150 µmol m^{-2} s^{-1}, 3 runs).

The net photosynthetic rate and dark respiration of *Kalmia latifolia* was overall lower than for 'Holsteiner Cox' in the same setup under the same lighting conditions (see Chapter 5.2.2). P_n averaged 0.4 µmol h^{-1} $plant^{-1}$ and did not surpass 1.4 µmol h^{-1} $plant^{-1}$. It

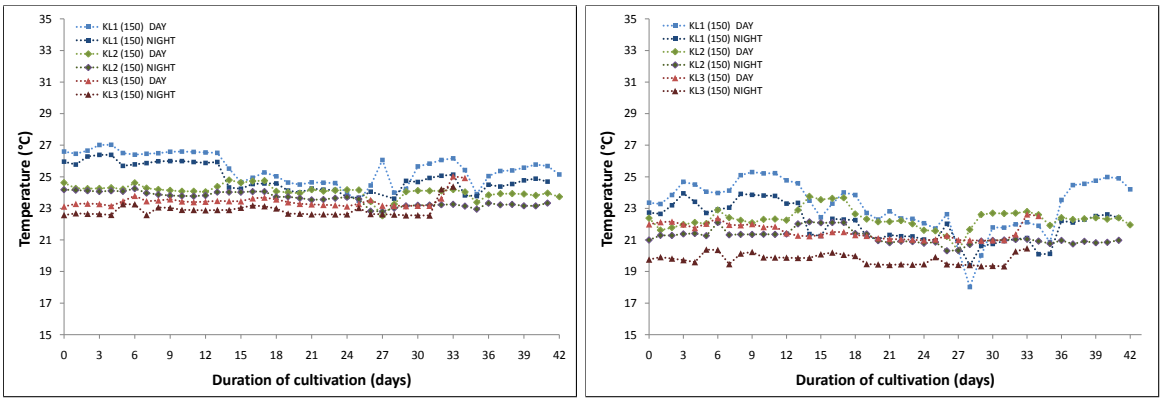
Tab. 5.7: Carbon dioxide concentration within PA-TIS for *Kalmia latifolia* in the second setup. a) first run; b) second run; c) third run

First run	(a) 
Second run	(b) 
Third run	(c) 

even was negative for some days of cultivation. The dark respiration remained also fairly constant throughout the *Kalmia* rooting (-0.6 µmol h^{-1} $plant^{-1}$).

5.3.3 Temperature

The mean temperature in the PA-TIS during the cultivation of *Kalmia latifolia* lay between 23 and 27 °C during the day. Higher temperatures between 26 and 27 °C were only recorded during the first run (KL1(150) in Fig. 5.31a) and lowered to 25 °C after 12 days of cultivation. During the third run (KL3(150)), the room temperature was low in the night (19.5 °C), which also resulted in the lowest vessel temperature during the night (< 23 °C). Overall, the variation of temperature was much lower in PA-TIS than in the room.



(a) (b)

Fig. 5.31: Mean temperature during the cultivation of *Kalmia latifolia*, divided into day and night temperature: a) in one of three PA-TIS vessels under a PPFD of 150 µmol m^{-2} s^{-1} and b) room temperature. 3 runs.

5.3.4 Relative humidity

The relative humidity in the PA-TIS ranged between a minimum 65 % during the night and 85 % during the day. For all three runs, the relative humidity during the day was higher than during the night. In all runs, the humidity was fairly constant with little variation.

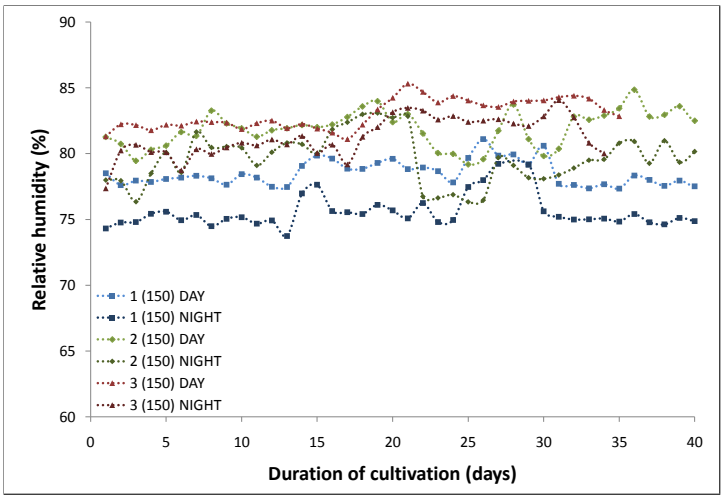


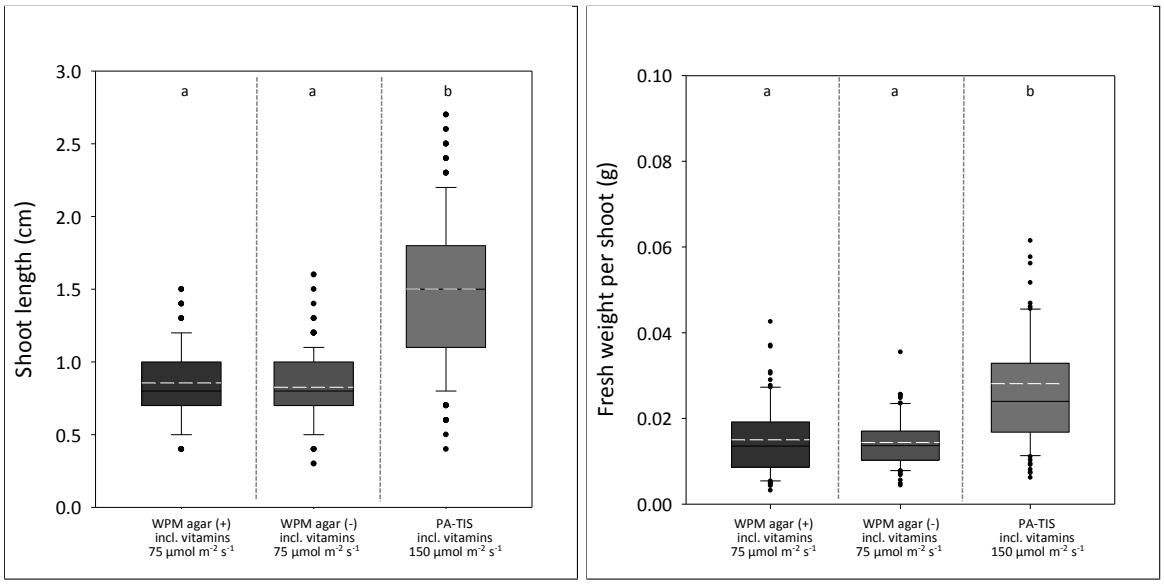
Fig. 5.32: Mean relative humidity during the cultivation of *Kalmia latifolia* under 150 µmol m^{-2} s^{-1}. 3 runs.

5.3.5 Plant quality and production of fresh weight

In preliminary tests on rooting of *Kalmia latifolia* conducted at the Tree Nursery Science Section, Leibniz Universität Hannover, the rooting efficiency of *in vitro* propagated shoots was very low. Additionally, a bacterial contamination of the plants became apparent when cultivating them on agar media (Fig. 5.34).

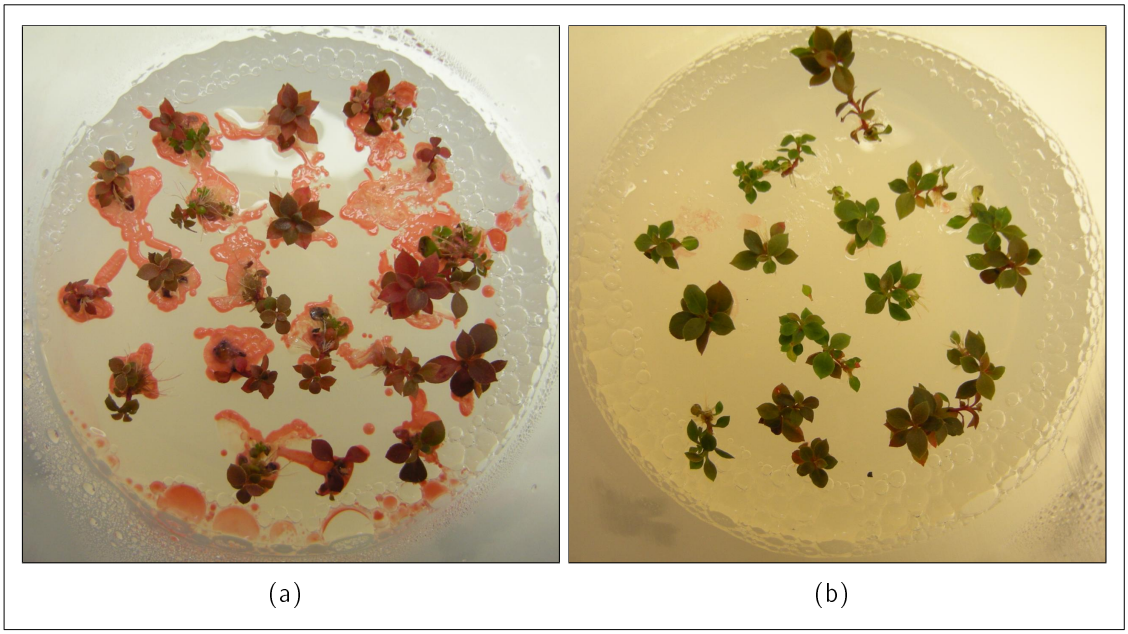
Shoots rooted on WPM agar (+) all had a red stem and reddish leaves, but when they were cultivated on WPM agar (-), the stem was brown and the leaves were green. In PA-TIS, the stem was also red, but the leaves were green (Fig. 5.35). The shoots were shorter on agar (0.8 and 0.9 cm, see Fig. 5.33) with few newly formed leaves, whereas they were elongated in PA-TIS with more leaves that were sometimes glassy (as in Fig. 5.35c). The shoot length of the plants cultivated in PA-TIS was significantly higher than of those on WPM agar (Fig. 5.33a).

When roots were formed by the shoots cultivated on WPM agar (+), they did not develop in the agar medium, but as air roots originating from the nodes or internodes, such as in Fig. 5.34a. On WPM agar (-), both air roots and roots that developed from callus tissue were detected (see Fig. 5.35b). The roots in PA-TIS sprouted mainly from the end of the shoot or the lowest internode without callus formation (Fig. 5.35c). A significantly higher accumulation of fresh weight was found for *Kalmia* in PA-TIS (Fig. 5.33b). It was on average 1.7 times higher than for the agar treatments. The dry weight was not determined since all plants were transferred *ex vitro*.



(a) (b)

Fig. 5.33: (a) Shoot length; (b) fresh weight of *Kalmia latifolia* after six weeks in control cultures (WPM agar (+)/(-)) or PA-TIS. Treatments labeled with the same letter do not differ significantly at $p \leq 0.05$ (Dunn's test). AM = white dotted line. 3 runs.



(a) (b)

Fig. 5.34: Bacterial contamination in *Kalmia* agar culture. a) WPM agar (+); b) WPM agar (-).

5.3.6 Rooting and acclimatization success

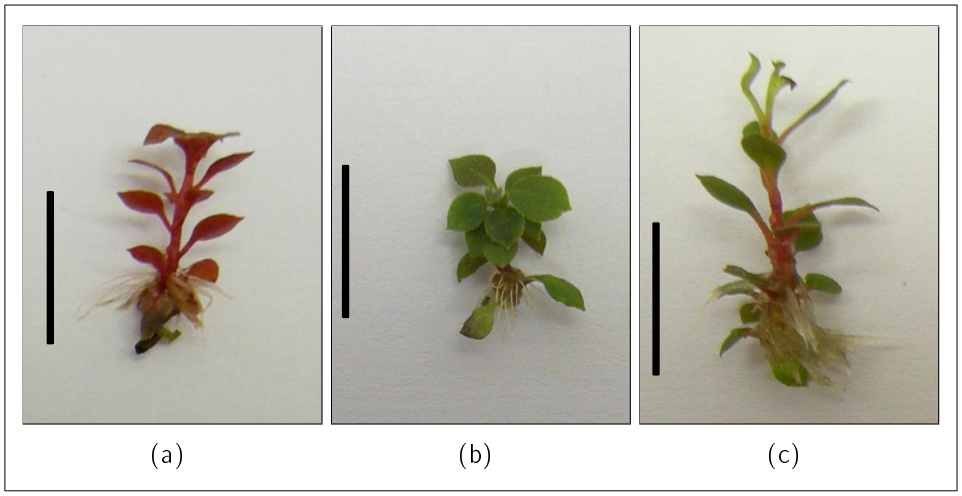


Fig. 5.35: *Kalmia latifolia* plants after 6 weeks of treatment. (a) WPM agar (+); (b) WPM agar (-); (c) PA-TIS. Black bar = 1 cm

The results of rooting *Kalmia* are summarized in Tab. 5.8. On WPM agar (+) the lowest rooting efficiency was achieved with 23 %. A higher number of rooted shoots was detected on WPM agar (-) (60 %), but the rooting was most successful in the PA-TIS with 89 %. The longest roots were also found in the PA-TIS (see Fig. 5.36). Of all rooted shoots, the ones on WPM agar (-) had the significantly shortest roots (Fig. 5.36). The number of roots was not recorded, because the roots were very small and thin, as can be seen in Fig. 5.35. The shoots were solely divided by their rooting intensity: strong (> 10 roots), weak (< 10 roots) and non-rooted (Tab. 5.8).

Tab. 5.8: Rooting success and intensity of *Kalmia latifolia* depending on treatment.

Treatment	**PPF**	**Rooting efficiency**	**N^{o} of shoots divided by rooting intensity**		
	$(\frac{\mu mol}{m^2 s})$	(%)	strong	weak	no
WPM (+)	75	23	21	22	141
WPM (-)	75	60	80	32	75
PA-TIS (-)	150	89	165	37	24

The *Kalmia* shoots were transferred *ex vitro* after the respective treatments in PA-TIS or on agar and planted according to the aforementioned rooting intensities (strong, weak and no rooting; Tab.5.8).

In Tab. 5.9, the acclimatization success after four weeks is summarized. Shoots rooted in PA-TIS had the highest survival rate (85 %) followed by WPM agar (-) and WPM agar

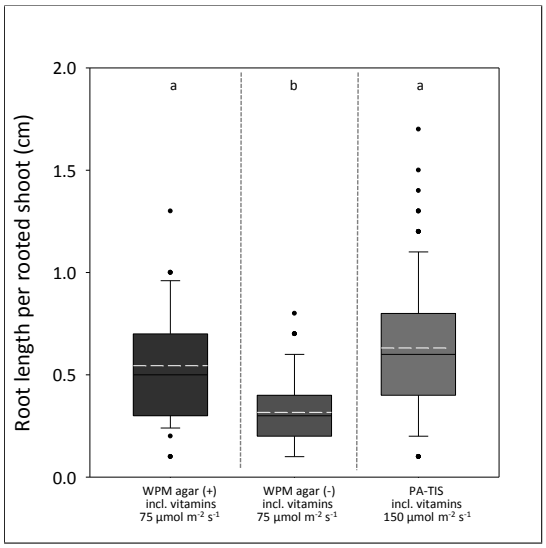


Fig. 5.36: Root length of rooted *Kalmia latifolia* shoots after six weeks in control cultures (WPM agar (+)/(-)) or PA-TIS. Treatments labeled with the same letter do not differ significantly at $p \leq 0.05$ (Dunn's test). AM = white dotted line. 3 runs.

Tab. 5.9: *Ex vitro* acclimatization success of rooted *Kalmia* shoots.

Treatment	**PPFD** (μmol m^{-2} s^{-1})	**Transferred ex vitro**	**Acclimatized after 4 weeks**	**Acclimatization** (%)
WPM agar (+)	75	128	79	62
WPM agar (-)	75	133	104	78
PA-TIS (-)	150	144	123	85

(+). The acclimatization success divided by the rooting intensity is depicted in Fig. 5.37. The highest loss of plants was recorded for WPM agar (+), when the plantlets were not rooted. For both other treatments, the acclimatization success did not depend so strongly on the rooting intensity with losses in any of the rooting intensities.

In Fig. 5.38 to 5.40, *Kalmia* shoots are presented four weeks after the transfer to the greenhouse. The strongly rooted plants originating from the PA-TIS were the furthest developed with the largest leaves and shoot height (Fig. 5.40a). Plants from both agar cultivations fared worse, having even smaller leaves than the non-rooted PA-TIS shoots. Many of the poorly and non-rooted plants from the agar treatments had already withered after four weeks (see Fig. 5.38b or 5.39b).

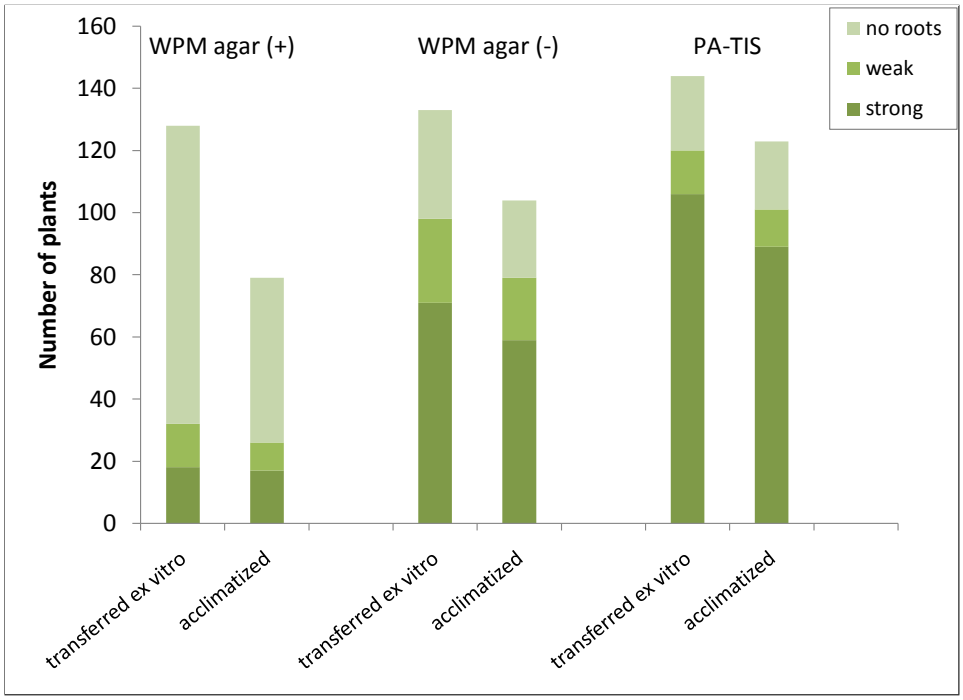


Fig. 5.37: Acclimatization success of *Kalmia latifolia* divided by treatment and rooting intensity (strong, weak, no roots).

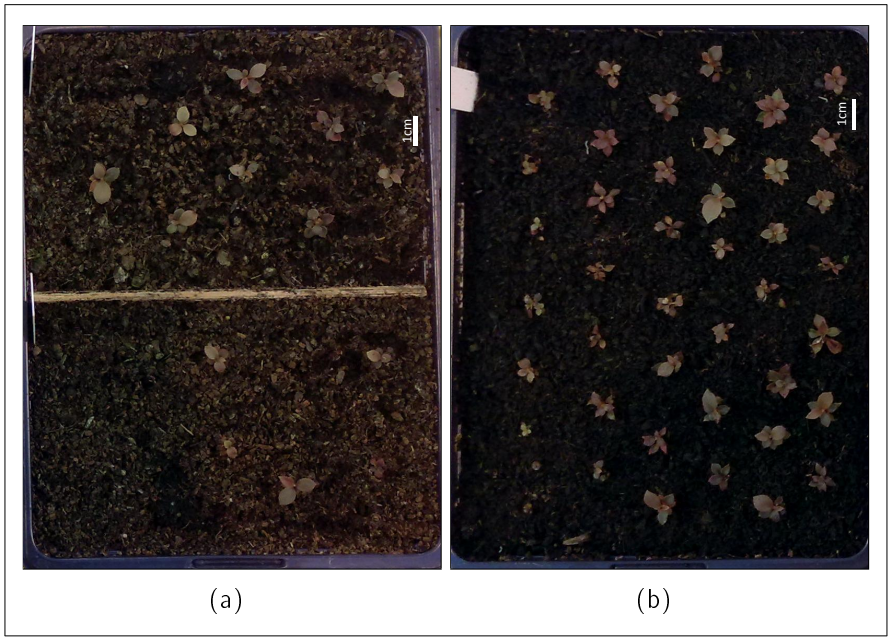


Fig. 5.38: *Kalmia* cultivated on WPM agar (+) after four weeks *ex vitro*, sorted by rooting intensity (a) strong (upper part) / weak (lower part); (b) non-rooted. White bar = 1 cm.

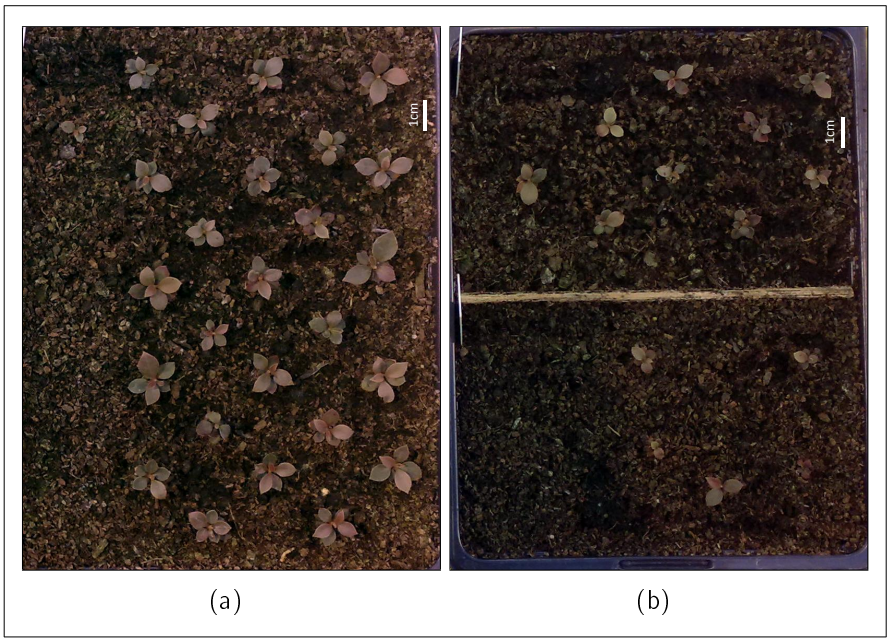


Fig. 5.39: *Kalmia* cultivated on WPM agar (-) after four weeks *ex vitro*, sorted by rooting intensity (a) strong; (b) weak (upper part) / non-rooted(lower part). White bar = 1 cm.

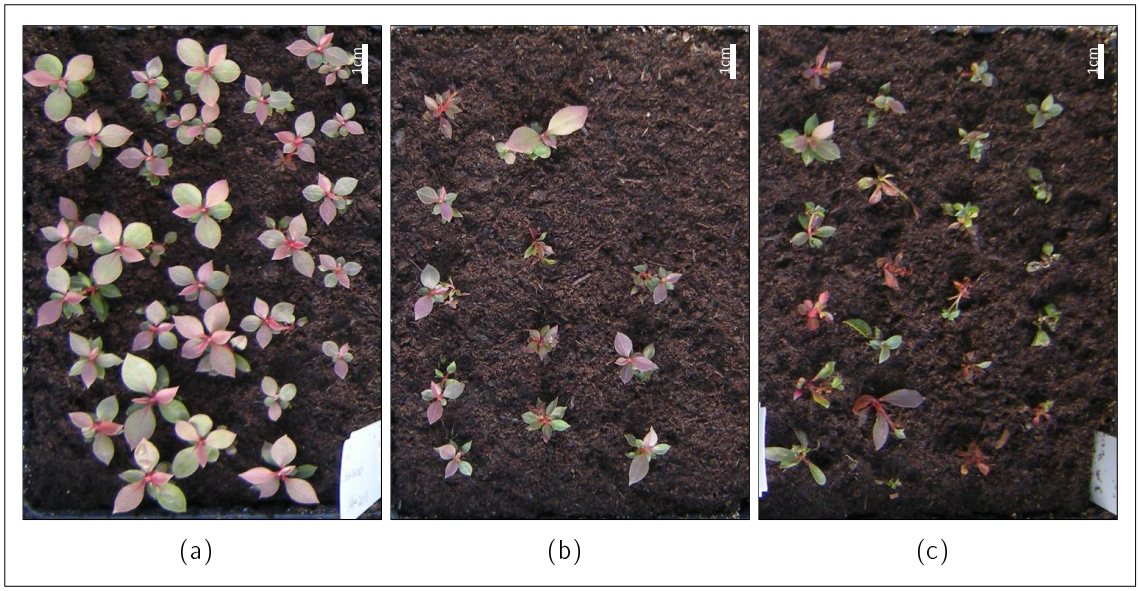


Fig. 5.40: *Kalmia* cultivated in PA-TIS after four weeks *ex vitro*, sorted by rooting intensity (a) strong; (b) weak; (c) no roots. White bar = 1 cm.

6 Discussion

The objective of the present study was the combination of a photoautotrophic cultivation with a temporary immersion culture. In order to evaluate the success of the establishment of this photoautotrophic temporary immersion system (PA-TIS) as well as the influence of the growth factors ventilation, lighting, relative humidity, temperature, and nutrient supply, different plants, namely *Malus* × *domestica* Borkh. cv. 'Holsteiner Cox' and *Kalmia latifolia* cv. 'Ostbo Red', were cultivated and rooted in the PA-TIS. The quality of the rooted shoots was compared to a conventional photomixotrophic culture on agar medium (MS agar (+)) and an agar culture without the addition of sugar (MS agar (-)). The net photosynthetic rate (only in PA-TIS) and stomatal aperture (only for 'Holsteiner Cox') were recorded as an immediate plant response to the different treatments and light intensities the plants were submitted to.

In the first section of this chapter, the results of the environmental control within the PA-TIS will be discussed. Afterwards, the impact of the regulated growth factors on the rooted shoots and their photosynthetic activity will be presented in detail and compared to other publications.

6.1 Environmental control

6.1.1 Carbon dioxide and ventilation

The addition of carbon dioxide to the vessel was regulated as described in Chapter 4.4.9. In the preliminary test, a different approach to measuring the carbon dioxide uptake had been chosen. The IR-CO_2/H_2O-analyzer (LI-7000) had two measuring cells for the estimation of carbon dioxide differences between gas streams. The difference between $CO_{2_{in}}$ (cell A) and $CO_{2_{out}}$ (cell B) were internally determined by the analyzer. The problem in using both cells was a significant delay between the measurement in them, even when they were directly connected. Also, a calibration difference became apparent in the pretests that could not be eliminated even by the producer LI-COR. Therefore, only one of the cells could be used and differences had to be recorded between two measuring points. The gas had to be circulated in a loop through the single cell and carbon dioxide was added as a pulse to the gas stream whenever the concentration dropped below 1200 µmol mol^{-1}.

This closed-loop ventilation of the PA-TIS proved to be problematic. As mentioned before, gas cannot be added to a closed vessel without inflating and finally bursting it. In the preliminary tests, a water seal had been added to the loop, but the resulting loss of cultivation air was higher than the one through the gaskets. That is why the mean carbon dioxide loss (c_{loss}) had to be recorded beforehand for both setups (Fig. 4.8, p. 53).

The course of carbon dioxide in Tab. 5.1c (p. 63) suggests that something was amiss with the ventilation of the third run under 115 + 75 µmol m^{-2} s^{-1}, because the carbon dioxide concentration dropped to approximately 300 µmol mol^{-1} on the 23rd day of cultivation and did not change for four days. This points to a blockage between the vessel outlet and IR-analyzer inlet, because at least the dark respiration would have resulted in an increase of the CO_2-concentration during the night. The reason for these results could be a clogged filter. Water vapor occasionally condensated in the tubes and filters when the bottom cooling did not lower the vessel temperature enough. Since the micro-filters (d = 0.2 µm were not water-permeable, water accumulated in them which led to a reduced gas stream. In the same run (Fig. 5.4, p. 67), the room temperature dropped below the PA-TIS' temperature during the night. This could explain the blocking of the filter, because lower temperatures in the connecting hoses than in the PA-TIS would result in water condensation therein. The net photosynthetic rate could not be determined from days 24 to 28 due to the lack of uptake data and is therefore not shown in Fig. 5.1 (P_n and R_d 3(115+75), p. 64). In Tab. 5.1e and f (p. 63), data is missing due to problems with the data logger. The regulation of the environmental control worked, and hence the provision of PA-TIS with carbon dioxide, but could not be recorded.

In the second setup, the problems were of a different nature. Three PA-TIS vessels were monitored consecutively with a single IR-analyzer by switching the gas streams, resulting in a 10 minute interval in which a single vessel was controlled. The IR-analyzer was not flushed in between the measurements, which resulted in a slight intermixture of the cultivation air. The effect can be seen in Fig. 4.8b for an empty vessel: even though no carbon dioxide was added to the PA-TIS vessel shown in this figure, the carbon dioxide concentration rose in between two measurement cycles. In this case, the carbon dioxide concentration in the antecedent PA-TIS was much higher than 3000 µmol mol^{-1}, so that the intermixture of the gas streams had a strong effect. Assuming that the concentration difference between vessels containing plants was not so high, the effect during the testing with plants would not be so distinct. Nonetheless, the first difference measurement after the switch was left out, so that eight measurements remained to determine P_n for a single interval. As mentioned before, only the first 22 days of cultivation are shown in

Tab. 5.3 and 5.4 (pp. 79 and 80), because Excel 2007 cannot display more than 32000 data points.

The valve connecting the carbon dioxide bottle to the second vessel in the second setup did not close properly, which resulted in a constant carbon dioxide provision to this PA-TIS vessel. The effect can be seen in Tab. 5.3a and 5.3d: the carbon dioxide concentration did only drop below 3000 µmol mol^{-1} a few times during the cultivation. The estimation of P_n was thus difficult (1-2(150) in Fig. 5.11 and 2-2(150) in Fig. 5.12, pp. 81 and 82). The valve was later replaced, so that the maximum measurable concentration was not surpassed for long periods of times anymore (Tab. 5.3f, p. 79).

The high carbon dioxide concentrations in Tab. 5.4 (p. 80), on the other hand, did not result from a leaking valve but from high respiration rates under the continuous lighting. The plants were submitted to a constantly high PPFD of 150 µmol m^{-2} s^{-1}, so that respiration under light took place. The carbon dioxide concentration was low in the later days of the second run under these conditions, because the CO_2-bottle was almost empty. When it was replaced, the concentration rose again.

Roels et al. (2006) monitored the headspace composition during the photomixotrophic multiplication of plantain in a temporary immersion bioreactor (TIB). Compared to a conventional culture on gelrite (semi-solid, 2.5 g l^{-1}), the carbon dioxide concentration in the TIB did not increase so strongly. The maximum concentration measured in the TIB was 5.7 % (57000 µmol mol^{-1}), but in the semi-solid even more carbon dioxide was produced (11.8 % = 118000 µmol mol^{-1}). These high increases were explained by high respiratory activity through sugar consumption and the lower concentration within a temporary immersion bioreactor (TIB) with the periodical exchange of the headspace during the immersion cycles. Escalona et al. (2003) showed a similar course for carbon dioxide in the photomixotrophic cultivation of pineapple in a TIB. The carbon dioxide concentrations within the bioreactor headspace differed strongly from those of a liquid culture. Under high PPFD (225 µmol m^{-2} s^{-1}), up to 14000 µmol mol^{-1} were measured in the liquid culture as opposed to 2000 µmol mol^{-1} in the TIB. The high concentration was explained by high respiration rates under light resulting from stress. Photomixotrophic pineapple plantlets cultivated in a TIB by González-Olmedo et al. (2005) reacted quite differently and took up carbon dioxide quickly, reducing the headspace concentration from 2500 to 1000 µmol mol^{-1} within an hour under a PPFD of 80 µmol m^{-2} s^{-1}. This demonstrates that even in photomixotrophic cultures the carbon dioxide consumption may be high, although sugar is provided as carbon source. A faster decrease of carbon dioxide should hence be expected for a photoautotrophic culture.

The regulation of carbon dioxide in the present study was not ideal. In other publications on photoautotrophic growth (see Tab. A.3, p. 178), carbon dioxide was mostly provided passively by increasing the carbon dioxide concentration of the cultivation room. Even in the publications on forced ventilation, the carbon dioxide enrichment occurred outside the vessel and the surrounding gas was pumped in and out of the vessel (Fujiwara et al., 1988; Kirdmanee et al., 1995b; Nguyen et al., 2000, 2001; Zobayed et al., 1999c, 2000a,b; Heo et al., 2001; Afreen et al., 2002a; Xiao and Kozai, 2004) . Since high amounts of carbon dioxide are needed for the elevation of the carbon dioxide concentration in a whole room from atmospheric (380 µmol mol^{-1}) to photoautotrophic high (Tichá et al. (1998): up to 20000 µmol mol^{-1}) concentrations, the addition of carbon dioxide to a closed system seemed more resource-friendly. The amount of carbon dioxide needed to elevate the concentration from 380 to 1200 µmol mol^{-1} depends, of course, on the size of the room. A small cultivation room (37.5 m^3) was compared to the three PA-TIS vessels used in the present study in Tab. 6.1.

Tab. 6.1: Elevation of carbon dioxide concentration of the cultivation air from 380 µmol mol^{-1} to 1200 µmol mol^{-1} in a cultivation room or three PA-TIS vessels.

	Dimensions (m)	**Volume** (m^3)	**Amount of CO_2 needed** (g)
Cultivation room	3 x 5 x 2.5	37.5	55.32
3 x PA-TIS		3 x $6.26 \cdot 10^{-3}$	0.028

Only a fraction of the carbon dioxide is needed when the vessels are directly supplied. The estimation of the carbon dioxide in Tab. 6.1 needed to enrich PA-TIS may be too low, because a bit of carbon dioxide was lost to the cultivation room through the gasket and hence "wasted". Nonetheless, the amount needed would still be much lower than for the enrichment of a whole room. The loss through the gasket cannot be prevented, if closed ventilation is to be maintained.

Since the carbon dioxide concentration within the PA-TIS vessel was constantly controlled, a source-sink analysis for carbon dioxide should be possible. Especially in the first setup, not all datasets are complete due to data logger problems so that a balancing of carbon dioxide uptake and loss was not possible.

Even in the second setup, only three of seven data sets for a PPFD of 150 µmol m^{-2} s^{-1} (16/8 h) are complete for both P_n and R_d, because during the night the carbon dioxide concentration in PA-TIS often rose above 3000 µmol mol^{-1} so that the respiration rate could not be determined properly. In Tab. A.9 (p. 183), the carbon dioxide data for the first vessel in the second run are summarized (2-1(150), see Tab. 5.3c for CO_2 and

Fig. 5.12 for P_n, pp. 79 and 82).

When speaking of the net photosynthetic rate, the assimilation of carbon dioxide through photosynthesis is meant. The basic equation for the photosynthesis, in which carbon dioxide is converted into glucose, is shown in Eq. 6.1.

$$6CO_2 + 6H_2O \rightarrow C_6H_{12}O_6 + 6O_2 \tag{6.1}$$

The molecular weight of carbon dioxide is 44.01 g mol^{-1}, whereas it is 180.16 g mol^{-1} for glucose. The CO_2:$C_6H_{12}O_6$ ratio equals 1.47, because six molecules of CO_2 are needed for building one molecule of glucose ($\frac{264.06}{180.16}$). The production of glucose via photosynthesis equals accumulation of biomass, dry matter in explicit.

In the cultivation described in Tab. A.9, both P_n and R_d were multiplicated by their duration (16 or 8 hours, respectively) and the number of shoots. Thereby, the amount of carbon dioxide taken up during the day and lost during the night was determined for each day separately (P_n + R_d). The amount of carbon dioxide assimilated in this cultivation in the PA-TIS was 3.22 g.

The accumulation of dry matter could only be extrapolated, because the dry matter content could not be determined in the beginning of the cultivation. The fresh weight of all shoots was 2.2 g at the beginning of the cultivation. The dry matter content after 4 weeks of cultivation was 17 %. Assuming that it did not change significantly throughout the cultivation, the dry weight of all shoots in the beginning would have been 0.37 g. At the end of the cultivation, the dry weight was 2.52 g. This would result in an accumulation of 2.15 g dry weight. The CO_2:$C_6H_{12}O_6$ ratio for this cultivation would then be 1.50, slightly higher than the theoretical assimilation.

The difference could be explained by the assumed DMC of 17 %. If the plants had a lower dry matter content at the beginning of the cultivation (e.g. 14.5 %), the result would have corresponded exactly to the ratio of 1.47 predicted above.

The continuous measurement of carbon dioxide in a closed cycle as it was demonstrated in this work can, in conclusion, not only be used for the estimation of the net photosynthetic rate, but also for the balancing of carbon dioxide sinks and sources *in vitro*. In order for such measurements to be successful, a working data logging system and limitations to the carbon dioxide provision to prevent overprovision (Tab. 5.3a, p. 79) have to be available.

6.1.2 Lighting

In the first setup, lateral lighting was applied to the PA-TIS additionally to overhead lighting. Kozai et al. (1992b) suggested that the distribution of light *in vitro* was not optimal with exclusive overhead lighting, because the upper leaves would shadow the lower leaves in the course of cultivation, resulting in suboptimal photosynthesis. When the lateral lighting was applied from two sides of the vessel in the present study the plants grew unevenly. The closer the plants were to the light sources, the bigger they were and the more roots they developed (Fig. 5.9, p. 76). It can be deduced that lateral lighting is beneficial for the shoots, but must be applied from all sides of the vessel in order to overcome locational disadvantages. In the present case, the construction did not allow the installation of further lamps since the vessel had to be placed under the lamps and the access to the PA-TIS (e.g. for the attachment of gas hoses, medium bottle etc.) would have been hindered by more lamps. Also, the vessel temperature would have been influenced significantly by such a high irradiance, so that additional cooling would have been necessary. An easier way to overcome locational disadvantages was to increase the PPFD overhead to 150 µmol m^{-2} s^{-1} by further lowering the distance between the lamps and PA-TIS as well as adding a reflective covering to the lamp mounting.

Throughout literature, the light intensities applied to the reference cultures were always kept lower than for the photoautotrophic culture. Zobayed et al. (1999c), for instance, chose a PPFD of 150 µmol m^{-2} s^{-1} for the photoautotrophic cultivation of sweet potato, whereas the photomixotrophic culture was kept under 60 µmol m^{-2} s^{-1}. In order to properly compare a photoautotrophic culture to conventional cultivation techniques, the conditions in the control have to be kept "normal". An increase of the PPFD in the control simultaneous to the increase in PA-TIS would have resulted in comparing photoautotrophic plants to photomixotrophic ones that were propagated under enhanced conditions. Since normally the PPFD in cultivation rooms does not surpass 75 µmol m^{-2} s^{-1}, this intensity was chosen in the present study.

The effect of lighting on the plant quality and photosynthetic activity will later be discussed.

6.1.3 Medium and PA-TIS immersion cycles

In the present study, three different cultivation approaches were chosen: a continuous immersion culture without supporting material, in which the plants (only 'Holsteiner

Cox') were in permanent contact with the medium, the PA-TIS, in which the plants were fixated in a carrier and periodically flooded with MS or WPM rooting medium (Fig. 4.2, p. 43) as well as media solidified with agar.

The immersion cycles proved to have an effect on the carbon dioxide concentration within the PA-TIS (Fig. 4.9, p. 55). Whenever an immersion was ended, the concentration within the vessels was lower than before the initiation. This can be explained by the PA-TIS' mode of operation depicted in Fig. 2.1 (p. 21). An air pump is used to displace the medium from the supply bottle to the cultivation vessel. The air used therefore was taken from the cultivation head space, since the aeration was conducted in a closed-loop. In order to overcome the water column posed by the medium and height difference as well, the pump had to build up a certain air pressure. If the air supply from the cultivation head space had not sufficed for generating this pressure (e.g. limitation of air flow through micro-filters), a depression would have been created within the vessel, resulting in suction of ambient air through the vessel's gasket. This could be an explanation for the carbon dioxide concentration drop. As long as the ventilation is maintained in a closed loop, low pressures can occur when air is forcedly moved.

6.1.4 Relative humidity

The relative humidity in the PA-TIS vessels was never higher than 90 %, lying between 60 and 85 % for most of the cultivations. Comparable results were achieved in other publications on photoautotrophic cultures (see Tab. A.3, p. 178). The overall low relative humidity was obtained by adding a bottom cooling to the PA-TIS. Egbers (2005) already showed that the addition of bottom cooling at 17 °C lead to a reduction of the relative humidity within cultivation vessels from 97 % to 85 %.

The third run under 115 + 75 µmol m^{-2} s^{-1} showed very different results in the course of relative humidity (Fig. 5.3a, p. 66), with a steady decrease of rH down to 20 %. For the same cultivation, a drop of the carbon dioxide concentration was detected (see Chapter 6.1.1). Since no cultivation air reached the IR-analyzer from the 23rd day on, the aforementioned blocking of the microfilter could also explain the decrease of the relative humidity. As mentioned above, the room temperature during the night was much lower than the temperature within the PA-TIS. A condensation of water in the connecting hoses would have resulted in a lower relative humidity and finally a blockage of the filter. A similar course was detected in the third run under continuous lighting (Fig. 5.15b,

p. 84). Again, a reduced carbon dioxide concentration was stated for the same cultivation (Tab. 5.4, p. 80). The drop of the relative humidity on the eighth day correlates with a decrease of carbon dioxide within the PA-TIS, hence a reduced gas stream.

For all cultivations, the mean relative humidity within the PA-TIS was higher during the day than during the night (Fig. 5.3 to 5.15, pp. 66 - 84). This correlates with the mean temperature that was also higher during the day than during the night. Higher temperatures lead to more evapotranspiration, which strongly influences the water content of the cultivation vessel. Under 115 + 100 µmol m^{-2} s^{-1} and 150 µmol m^{-2} s^{-1}, a slight increase of the relative humidity throughout the cultivation was recorded. This may be attributed to the increasing biomass within the PA-TIS, because a higher leaf area results in higher transpiration rates (Zobayed et al., 2001a).

6.1.5 Temperature

The temperature in the PA-TIS lay between 24 and 28 °C most of the time. The average temperatures for all cultivations are summarized in Tab. A.11 (p. 184). No DIF-treatment was applied in the present study. Nonetheless, a slight difference between day and night temperatures was measured due to the influence of the lighting on the vessel temperature. The temperature increase thereby was approximately 1 K. The mean temperature in the second setup (Fig. 5.16, p. 85) was slightly higher than in the first setup under 115 + 75 µmol m^{-2} s^{-1} even though air conditioning was applied.

Following factors proved to have an influence on the PA-TIS temperature:

- Room temperature: Not only did the room temperature strongly influence the PA-TIS temperature, but also did electrical devices increase the room temperature (especially the fluorescent lamps). The difference becomes apparent in the air-conditioned room when comparing day and night temperatures (Fig.5.16b, p. 85)
- Lighting: The temperature during the day was always higher than during the night. The difference of ± 1 K can be reduced to the lighting since nothing else was changed in the setups.
- Magnetic valves: In the second setup, the gas stream to be measured in the IR-analyzer was regulated by magnetic valves. These were opened for 10 minutes during the measuring cycle and heated up after some time.
- Pumps: The gas was continuously pumped through the system. The influence of the pumps may be small, but should be considered for the sake of completeness.

- Bottom cooling: As shown in Fig. 5.4 (p. 67), the bottom cooling functioned not only as means of reducing the relative humidity, but also as cooling device.

6.2 Net photosynthetic rate

The net photosynthetic rate was calculated according to Equation 4.5. The combined volume of the box used for the PA-TIS and all connected hoses was used as volume (V) in this calculation (Tab. 4.6, p.42). Nguyen et al. (2001) also used the 'Bio-Safe Carrier' as cultivation vessel for coffee plantlets, but the volume was not correctly determined. Instead of using the vessel's inner dimensions, the producer specifications were used (37 x 18.4 x 17 cm). These dimensions refer to the actual vessel size including handles and rims. Also, the vessel was not rectangular, but had a reduced bottom and lid area. By flooding the box completely with water, the actual volume was determined in the present study to be 6.13 l and not 11.14 l. The P_n values estimated by Nguyen et al. (2001) would accordingly be 1.8 times too high. The maximum P_n they recorded after 40 days was 16 µmol CO_2 h^{-1} $plant^{-1}$. Dividing the value by 1.8 would result in a much lower photosynthetic activity (8.9 µmol CO_2 h^{-1} $plant^{-1}$) that would not be higher than the control (around 9 µmol CO_2 h^{-1} $plant^{-1}$). Interestingly, Heo et al. (2001) and Wilson et al. (2001) likewise specified the higher volume (11.14 l) in their respective studies, but did not use it to calculate P_n. Zobayed et al. (2004a) gave an overview on all cultivation vessels used for large-scale photoautotrophic cultivation. Citing the aforementioned publications, an even higher volume of the box was specified (12.8 l). No explanations were given on how this value was calculated.

Under a 16/8 h light/dark regulation of lighting, an increase of P_n was detected for all treatments of 'Holsteiner Cox' and *Kalmia latifolia* throughout the cultivation, independent of the applied PPFD. This increase differed to a greater or lesser extent between runs under the same lighting conditions (e.g. see second run in Fig. 5.1, p.64). A mean value for all runs and repetitions under the same condition was hence calculated for better comparability and is shown in Fig. 6.1 for 'Holsteiner Cox' and 6.2 for *Kalmia*. In both figures, P_n was approximated with a sigmoidal trend. The results of this approximation are summarized in Tab. A.4, p. 179. Surprisingly, Mei-Lan et al. (2003) used a different approach and applied two different polynomic approximations (second or third order) for the description of P_n in their study on photoautotrophic cultivation of *Lilium* bulblets. The choice was not explained and seemingly only depended on the best R-square value.

In other publications, the approximations between the measurements were not described at all (Afreen et al., 1999; Nguyen and Kozai, 2001a; Lian et al., 2002; Xiao et al., 2003; Aragón et al., 2005; Xiao and Kozai, 2006), but most curves followed a logistic progression.

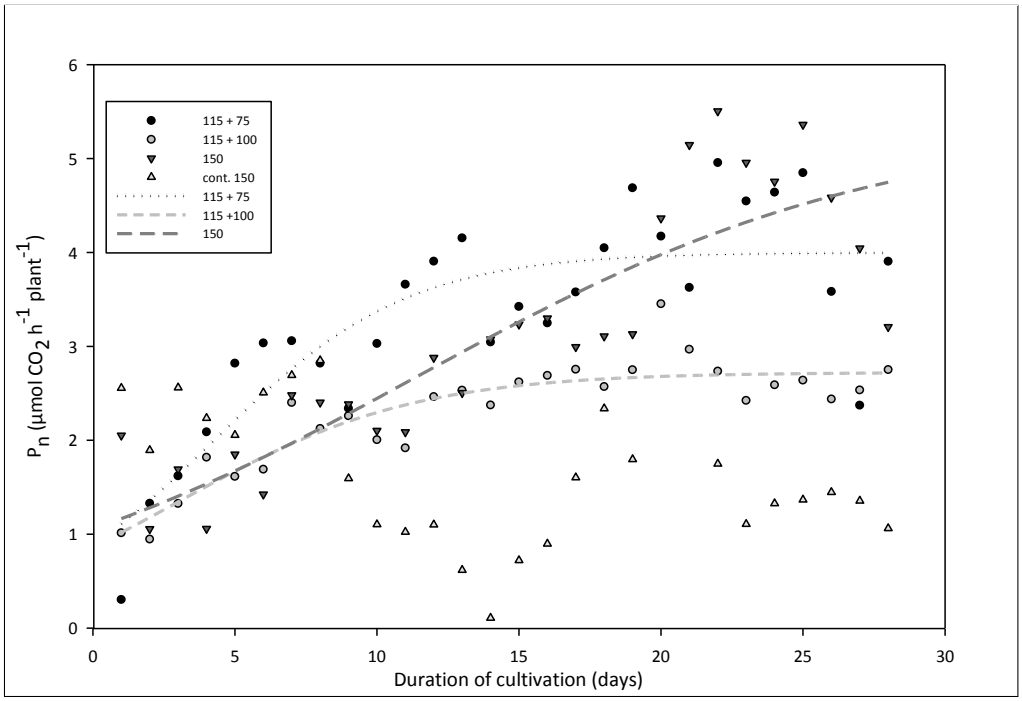


Fig. 6.1: Mean net photosynthetic rate (P_n per plant) for all treatments of 'Holsteiner Cox' in PA-TIS, grouped by PPFD (µmol m^{-2} s^{-1}), sigmoidal trend.

Even though most P_n-courses under the same lighting conditions differed strongly from each other, mean values were determined in order to compare the different treatments. The results are combined in Tab. A.8 (p. 182). These values were used to create Fig. 6.1. The P_n mean values for any of the cultivation did not surpass 6 µmol h^{-1} $plant^{-1}$. For a better visualization, a sigmoidal trend was added, although it could not fully describe the P_n course (for approximation and R^2 see Tab. A.4, 179). For all three treatments with 16 h light period and 8 h dark period, P_n rose during the cultivation. While it almost stagnated for plants grown under 115 + 100 µmol m^{-2} s^{-1}, it reached a peak for 115 + 100 and 150 µmol m^{-2} s^{-1} on the 22nd day, whereupon it slightly decreased in both cases. This development unfortunately cannot be shown by the sigmoidal trend. Plants cultivated under continuous lighting showed an unusual P_n trend with high fluctuations. No sigmoidal approximation was possible for this treatment due to the unusual progression: In the first days of cultivation, P_n was higher than for all other treatments, but later decreased to almost 0 µmol h^{-1} $plant^{-1}$ (and below for single treatments, as seen in Fig. 5.14, p. 83), then rising again to an almost constant 1.5 µmol h^{-1} $plant^{-1}$. For

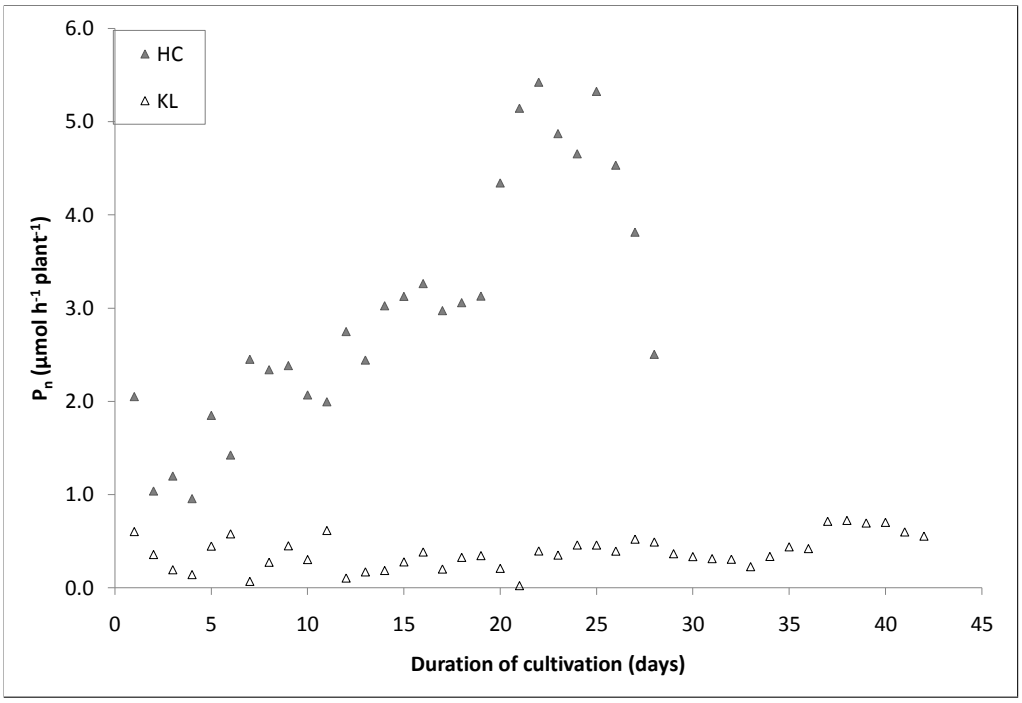


Fig. 6.2: Mean net photosynthetic rate (P_n per plant) for *Kalmia latifolia* (KL) in comparison to 'Holsteiner Cox' (HC) in PA-TIS under the same high PPFD (150 µmol m^{-2} s^{-1}, 3 runs).

most of the time, the highest P_n was achieved under 115 + 75 µmol m^{-2} s^{-1}, but around the 20th day, plants grown under 150 µmol m^{-2} s^{-1} had a higher photosynthetic activity. Both other treatments show a much lower P_n, never surpassing the two best treatments after the 8th day of cultivation.

The net photosynthetic rate of *Kalmia latifolia* was much lower than of the *Malus* shoots rooted in PA-TIS as shown in Fig. 6.2. In the first days of cultivation, P_n was even negative at times (Fig. 5.30, p. 101). The low photosynthetic activity did not result from carbon dioxide deprivation since the same CO_2-regulation was used for both plants cultivated in PA-TIS. The *Kalmia* shoots were much smaller than 'Holsteiner Cox' and had a very small leaf area (see Fig. 4.2, p. 43, for size comparison). The fresh weight of *Kalmia* was 15 times lower after a 6 week cultivation in PA-TIS (26 ± 12 mg) as opposed to 402 ± 186 mg of 'Holsteiner Cox' after 4 weeks under the same conditions. Afreen et al. (2002a) obtained equally low P_n values for coffee shoots developed from somatic embryos. P_n did not rise over 1 µmol CO_2 h^{-1} $plant^{-1}$ after 45 days, which was also attributed to the plants' small size.

The use of [µmol CO_2 h^{-1} $plant^{-1}$] as unit for the net photosynthetic rate is thus not ideal. A relation of P_n to the actual plant weight (fresh or dry) would only be realized if

the plants were actually weighed. A daily estimation of the fresh weight throughout the cultivation, however, would have influenced the microclimate of the vessel significantly. Additionally, the plants would have had to be cleaned and wiped dry before each weighing, which would have resulted in removing the nutrients from the plantlets' surface. Since other studies also resorted to expressing the net photosynthetic rate with [µmol CO_2 h^{-1} $plant^{-1}$] when non-destructive measurements took place (Afreen et al., 1999; Kozai et al., 1996; Nguyen and Kozai, 2001a; Xiao and Kozai, 2006; Xiao et al., 2003), it will be used in the following discussions for the sake of comparability.

In the following sections, the influence of different growth factors on the net photosynthetic rate will be discussed.

6.2.1 Influence of carbon dioxide and ventilation on the net photosynthetic rate

For the cultivation of 'Holsteiner Cox', the addition of carbon dioxide to the PA-TIS resulted in photosynthetically active plants. Under all lighting conditions, P_n was positive most of the time, so that carbon dioxide could be assimilated. The carbon dioxide concentration was monitored continuously in both setups in order to determine P_n for every day of the cultivation. For most days P_n was recorded unless the data logger did not work properly (see Tab. 5.1f, p.63).

In most publications on photoautotrophy, P_n was not measured daily, but only a few times throughout the cultivation. Xiao and Kozai (2006), for instance, took a carbon dioxide sample every five days and determined the CO_2-concentration by gas chromatography. Normal cultivation vessels with permeable lids were used for the multiplication of statice and only the carbon dioxide concentration in the culture room was elevated to 1500 µmol mol^{-1}. P_n was estimated as µmol CO_2 per hour and clump, with a clump equaling approximately 3 to 7 shoots. After 25 days of cultivation, P_n ranged between 12 and 25 µmol h^{-1} $clump^{-1}$, corresponding to a P_n of 2.4 to 7 µmol h^{-1} $plant^{-1}$. Both supporting material and BAP-concentration in the medium proved to have an influence on the net photosynthetic rate. Similarly, Kozai et al. (1996), Lian et al. (2002) and Nguyen and Kozai (2001a) determined P_n only every seven days by taking samples with a syringe. Aragón et al. (2005) used a leaf cuvette every seven days to measure P_n in a photomixotrophic TIS culture of *Musa* AAB. Only after transferring the plants *ex vitro* did P_n increase from approximately 5 µmol m^{-2} s^{-1} to more than 10 µmol m^{-2} s^{-1} (other

unit than the one used in this study, referring to the uptake per leaf area and second). This approach is quite laborious, because the plants had to be removed from the vessel every week in order to be monitored with the leaf cuvette.

In the present study, the online monitoring of carbon dioxide was successful and resulted in courses such as depicted in Fig. 4.6 (p. 49). The partitioning described in Chapter 4.4.3 was chosen to clarify the carbon dioxide course during day and night. During the day (sections 4 - 27) the uptake was high, resulting in a high decrease of the CO_2-concentration. The concentration also dropped during the night due to vessel leakage (partitions 1, 3 and 29), but the slope was not as steep as during the day. Also, the carbon dioxide concentration temporarily rose (sections 2 and 28). Between partitions 2 and 3 as well as between 28 and 29, the concentration reached a maximum before declining again. This maximum lay around 2000 µmol mol^{-1} in both cases. The course can be explained by two opposite carbon dioxide reactions: on the one hand, carbon dioxide is produced by respiration, on the other hand it is lost due to the permeability of the vessel. When the respiration rate R_d is high, the effect of $CO_{2_{loss}}$ is lower than of the production, therefore the concentration in the vessel rises. As soon as the production rate decreases, the leakage predominates and the carbon dioxide concentration decreases.

Morini and Melai (2005) also recorded the carbon dioxide concentration within cultivation vessels, but no CO_2-enrichment was applied. They divided the carbon dioxide balance of a photomixotrophic *Prunus cerasifera* culture into three phases: during the first five days of cultivation, the carbon dioxide production through respiration was higher than the uptake through photosynthesis. The next five days, the uptake was higher than the production, resulting in low carbon dioxide concentrations during the day. In the third phase, the production and uptake were similar and constant. This speaks for a logistic P_n-curve, since in the beginning the uptake would be low, in the middle the increase would be high and in the end P_n would stagnate. This correlates to the stagnation phase also detected in Fig. 5.1 to 5.2 and 5.11 to 5.13 (pp. 64 - 65 and 81 - 83), so that the mean values under discontinuous lighting also stagnated sooner or later (see Fig. 6.1, p. 119). Similar to these observations, the P_n did not increase in the cultivation of *Limonium* (Lian et al., 2002). Independent of the PPFD (100 or 200 µmol m^{-2} s^{-1}), P_n did not rise in the last week of cultivation. As mentioned above, in the present study P_n even decreased for some runs in the last days of cultivation. Nguyen and Kozai (2001a) obtained similar results in the cultivation of banana, where P_n dropped from a maximum 5 µmol CO_2 h^{-1} $plant^{-1}$ on day 21 to 3.5 µmol h^{-1} $plant^{-1}$ on day 28 under high ventilation rates. This decrease was explained by a reduction of PO_4^{3-} ions in the culture medium. This will be further

discussed in Chapter 6.2.3.

In the present study, high fluctuations between P_n values recorded throughout the cultivations may be explained by the method of carbon dioxide application to the vessel. The carbon dioxide concentration was regulated not to drop under 1200 µmol mol^{-1}. The concentration therefore varied between 1200 and ideally 3000 µmol mol^{-1} (maximum measurable value for the IR-analyzer). Nguyen et al. (1999a) showed for coffee plants that P_n highly depends on the carbon dioxide concentration within the vessel. An increase of carbon dioxide from 900 to 1700 µmol mol^{-1} resulted in doubling P_n from 2 to 4 µmol m^{-2} s^{-1}. In Fig. 5.1 (p. 64) the lowest P_n values of the second run (day 9 and 14) coincide with the lowest mean carbon dioxide concentrations within the vessel. For the third run, however, the highest carbon dioxide concentration in the PA-TIS was recorded on the same day as a decrease of P_n (day 21). Kozai et al. (1992a) predicted a saturation of P_d (net photosynthetic rate per leaf dry weight) for carbon dioxide concentrations higher than 1200 µmol mol^{-1} in a photoautotrophic cultivation of potato plants. All higher concentrations would thus have no positive influence on the photosynthetic activity. If this were the case, the concentration increase above 1200 µmol mol^{-1} in the present study would not have influenced P_n in any way.

It may be concluded that low carbon dioxide concentrations (under 1200 µmol mol^{-1}) can influence the net photosynthetic rate negatively, but low P_n do not exclusively result from carbon dioxide shortage. Other factors will be discussed in the following chapters.

6.2.2 Influence of lighting on the net photosynthetic rate

Additionally to a high carbon dioxide concentration, a high light intensity is needed for photoautotrophic cultivation *in vitro*. In the present study, different PPFD were applied. In the first setup, only used for the cultivation of 'Holsteiner Cox', lateral and overhead lighting was provided by fluorescent lamps. An increase of the overhead lighting from 75 to 100 µmol m^{-2} s^{-1} did not result in a higher net photosynthetic rate (Fig. 6.1, p.119). In this case, an interference of different growth factors may have led to a decreased P_n. The elevated light intensity coincides with an increase of the vessel temperature described in Chapter 5.1.4. The effect of temperature on P_n will be discussed later.

When the PPFD was further increased to 150 µmol m^{-2} s^{-1} in the second setup (only overhead, see Chapter HC2, p. 78), P_n was higher than for 115 + 100 µmol m^{-2} s^{-1}, but still lower than under 115 + 75 µmol m^{-2} s^{-1}. At the same time, the medium

composition was changed so that, again, the interplay of different factors influenced P_n negatively. For sugarcane, Xiao et al. (2003) determined P_n on three days of the cultivation (day 3, 10 and 17). The plants were most photosynthetically active under a PPFD of 400 µmol m^{-2} s^{-1} and a high carbon dioxide concentration (1500 µmol mol^{-1}) with a maximum P_n of about 90 µmol h^{-1} $plant^{-1}$. When low or high PPFD were paired with low ventilation rates, P_n did not increase at all. Sugarcane is a C_4 plant and can hence accumulate carbon dioxide more efficiently than C_3 plants such as *Malus* or *Kalmia*. The ten times higher photosynthetic activity can thereby be explained (*Malus*: max. 9.4 µmol h^{-1} $plant^{-1}$). Under photoautotrophic conditions, the net photosynthetic rate of *Lilium* bulblets also increased with rising light intensities (30, 60 and 120 µmol m^{-2} s^{-1}). After 40 days of cultivation a maximum P_n was achieved (6.8 µmol CO_2 h^{-1} $plant^{-1}$), which was comparable to the rates determined in the present study (Fig. 5.13, p. 83). Similar to Fig. 6.1 (p. 119), Lian et al. (2002) detected a stagnation of the net photosynthetic rate under all lighting conditions tested (50, 100 and 200 µmol m^{-2} s^{-1}). This phenomenon seems to occur independent of the light intensity and a probable cause, the substrate limitation, will be discussed in the following.

The most interesting effect of lighting on P_n was found under a continuous lighting with 150 µmol m^{-2} s^{-1} (Fig. 5.14, p. 83). The determination of P_n was not easy, because the highest concentration measurable with the IR-analyzer, 3000 µmol mol^{-1}, was often exceeded. The course of P_n strongly varied between the different runs. Overall, a slight decrease of photosynthetic activity throughout the cultivation could be stated (see Fig.6.1, p. 119). Even though there is no evidence that the respiratory TCA cycle cannot take place at the same time as photosynthesis, high carbon dioxide concentrations and high light intensities promote photosynthesis and 'dark' respiration is suppressed (Lawlor, 2001, pp. 206 - 207). Without dark periods and hence a functioning TCA activity, ATP and amino acid synthesis are inhibited. If ATP is not properly produced, photosynthesis cannot take place, and therefore P_n decreases.

Not only do the intensity and duration of lighting have an effect on the net photosynthetic rate, but also the composition. Sivakumar et al. (2006) tested different combinations of LEDs on a photomixotrophic sweet potato culture. An intermittent treatment with a combination of blue and red LEDs resulted in a significantly higher photosynthetic activity. This provides the ground for future studies on photoautotrophic cultivation with optimized LED-lighting.

6.2.3 Influence of medium and supporting material on the net photosynthetic rate

Of the three different cultivation types, namely continuous immersion, PA-TIS and agar culture, in which the 'Holsteiner Cox' shoots were rooted, only the PA-TIS was monitored for the photosynthetic activity of the plants. The influence of the supporting material (none in the continuous immersion culture, a carrier in PA-TIS (Fig.4.2, p. 43) or agar) on the net photosynthetic rate can therefore not be determined for the present study.

Afreen et al. (1999) on the other hand measured P_n of sweet potato plantlets grown on different materials: agar, gellan gum, vermiculite, sorbarod and florialite. The carbon dioxide probes were taken on days 7, 13, 18, and 22. Under all conditions, P_n stagnated after day 17. The highest activity, 13 µmol CO_2 h^{-1} $plant^{-1}$ (day 22), was achieved on florialite, the lowest on agar. Similarly, a higher P_n was measured for sweet potato grown on a mixture of vermiculite and paper pulp as compared to agar (Afreen-Zobayed et al., 2000). Xiao and Kozai (2006) tested different combinations of medium containing BAP with agar or florialite. The highest net photosynthetic activity was, again, obtained on florialite when the BAP-concentrations were low. In all publications it was concluded that the higher the porosity of the supporting material was the better the plants reacted and the more photosynthetic activity was recorded. In the present study, the plants in the PA-TIS were not placed into any supporting material, but only into a carrier. The accessibility of gas in the rootzone was hence much higher than in the agar cultures. Zobayed et al. (1999c) also indicated a positive effect of temporary immersion on the net photosynthetic rate of sweet potato plants. P_n was higher under a temporary medium provision of the rootzone than under a stagnant nutrient provision.

When the medium composition for 'Holsteiner Cox' was changed from MS medium containing vitamins to MS without vitamins, a decrease of P_n could be detected (Fig. 6.1, p. 119). Even though the lighting was additionally increased, P_n was mostly lower throughout the cultivation. Since the fresh weight of the shoots was significantly reduced by the vitamin elimination (see Tab. A.12, p. 185), the reduced photosynthetic activity is mainly connected to the lower leaf area of the smaller shoots.

The courses of P_n for plants grown under 16/8 h light/dark shown in Fig. 6.1 (p. 119) resemble a growth curve under substrate limitation. In fact, the medium was not changed throughout the cultivation of either 'Holsteiner Cox' or *Kalmia latifolia*, so that an exhaustion of nutrients is likely. Such a stagnation of the net photosynthetic rate was also

detected by Nguyen and Kozai (2001a). The reduction of PO_4^{3-} ions in the medium was made responsible for a reduced P_n. Since no nutrient concentrations were determined by Nguyen and Kozai (2001a), a publication by Kozai et al. (1995) was quoted where, in fact, a reduction of phosphate had been measured.

Phosphate is essential for building ATP; hence, a depletion of it would result in a low carbon dioxide assimilation rate. When the apple rootstock 'M9 EMLA' was propagated in TIS by Chakrabarty et al. (2007), phosphate and ammonium were quickly taken up. After 28 days, only 20 % of the phosphate and 25 % of the ammonium remained in the medium. Under continuous immersion, the uptake was even higher. Unfortunately, P_n was only recorded by Chakrabarty et al. (2007) on the last day of cultivation, so that a correlation between the nutrient and P_n development over the cultivation cannot be obtained.

It can be concluded that the provision of the plants with medium has an important impact on the photosynthetic activity. Not only does the medium influence the growth rate and hence the leaf area available for photosynthesis, but also the nutrients necessary to perform at least parts of the carbon dioxide assimilation.

6.2.4 Influence of relative humidity on the net photosynthetic rate

In closed *in vitro* vessels, the relative humidity is mostly very high due to evaporation of the medium and low gas exchange rates. This may lead to reduced transpiration rates of the propagated plants (Vanderschaege and Debergh, 1987). The relative humidity in the present study was only measured in the PA-TIS vessels and did not surpass 90 %. In the third run under 115 + 75 µmol m^{-2} s^{-1}, the lowest humidity of all treatments was measured (Tab. A.10, p. 184). The constant decrease of the relative humidity throughout the cultivation did not have an apparent positive or negative influence on the net photosynthetic rate. Since the difference of the relative humidity was not high between the other treatments, an immediate effect on the net photosynthetic rate cannot be determined.

6.2.5 Influence of temperature on the net photosynthetic rate

When the light intensity in the first setup (Fig. 4.3, p. 45) was increased to 115 + 100 µmol m^{-2} s^{-1}, the net photosynthetic rate did not increase as it would have been

expected. The room temperature during this treatment was the overall highest, resulting in high temperatures within the PA-TIS that temporarily lay over 30 °C (Fig. 5.5, p. 68). In all of the three runs under these conditions, P_n did not rise over 4 µmol CO_2 h^{-1} $plant^{-1}$. Nguyen et al. (1999b) stated that the leaf temperature of *Coffea arabusta* should not surpass 28 °C since it inhibits growth and influences P_n negatively. When the temperature optimum of the plant is exceeded, photosynthesis decreases in favor of photorespiration, because RuBisCO loses its affinity for carbon dioxide (McDonald, 2003, pp. 124 - 127). Additionally, stomata close in order to prevent water loss. Since neither 'Holsteiner Cox' nor *Kalmia* are C_4 plants, which have a much higher temperature optimum, temperatures above 30 °C would have a negative influence on carbon dioxide uptake. A temperature effect can also be seen in Fig. 5.11 to 5.13 (pp. 81 to 83). In the first run under 150 µmol m^{-2} s^{-1} the temperature was high during the day and the night (1(150) in Fig. 5.16a, p. 85). The net photosynthetic rate under the same conditions was low (Fig. 5.11, p. 81). During this run, the temperature was lowered from 26.5 °C to 24.5 °C on day 14; simultaneously P_n in Fig. 5.11 increased in both PA-TIS vessels from around 0 µmol CO_2 h^{-1} $plant^{-1}$ to positive P_n-values. The further the temperature was decreased, the higher the photosynthetic activity was (Fig. 5.16, p. 85): the mean day temperature was 24 °C in the second run, with higher P_n-values than in the first run, and 23 °C in the third run, corresponding to the highest course of P_n measured.

Interestingly, most *Malus in vitro* cultures are conducted at 25 °C or higher (Zimmermann, 1983; van der Krieken et al., 1993; Pasqual et al., 2000; Szankowski et al., 2003; Vieira et al., 2007). Riechers (1993) also propagated *Kalmia* at 25 ± 1 °C, whereas Flemer (1949) and Pavingerová and Šedivá (1999) applied 22 °C.

Lowering the temperature to 23 ± 1 °C in the present study resulted in a higher photosynthetic activity of the shoots. For the photoautotrophic cultivation of shoots, a temperature reduction of either the room or gas temperature should be considered. High PPFD lead to increasing leaf temperatures which have to be cooled down in order to promote photosynthesis (McDonald, 2003, p. 124). The bottom cooling may be a means of overcoming such high temperatures *in vitro*.

6.3 Plant quality - fresh weight, dry weight, leaves and stomata

The *Malus* and *Kalmia* shoots produced under the different conditions are presented in Fig. 5.6/5.18 and 5.35, respectively (pp. 70, 88 and 106). Only 'Holsteiner Cox' plantlets were tested in a continuous immersion culture, since the quality of the shoots after 4 weeks was very poor. The plants were hyperhydric and did not develop any roots, so that they could not be used for an *ex vitro* transfer. Only three repetitions were hence conducted for this cultivation type. Just as by Chakrabarty et al. (2007), the lowest dry matter content (DMC) was detected when continuous immersion was applied to the shoots. The recorded DMC of 10.3 % for MS continuous immersion (+) and 8.6 % for MS continuous immersion (-) matches their result for the apple rootstock 'M9 EMLA' of 8.8 %. Damiano et al. (2003), on the other hand, detected a DMC of almost 18 % for 'Jork 9' plantlets propagated in a liquid culture. In the temporary immersion cultures, they recorded both the lowest and highest DMC (2.5 and 21 %, respectively), depending on the length of the immersion cycles. In the present study, only the highest PPFD (150 µmol m^{-2} s^{-1}) resulted in a homogenous plant quality and high rooting efficiency for 'Holsteiner Cox' in PA-TIS. Therefore, *Kalmia* was only tested on agar and in PA-TIS under the conditions that proved to be successful with 'Holsteiner Cox'.

The overall quality differed strongly between the various treatments for both *Malus* and *Kalmia*. The 'Holsteiner Cox' shoots were cultivated with medium containing vitamins as well as medium without vitamins. Contrary to expectations, the best and most homogeneous shoot quality with a concurrently high rooting efficiency was obtained when the plants were cultivated on MS agar (+) including vitamins (Fig. 5.6b, p. 70). No hyperhydricity of the shoots was detected on MS agar (+), although it is often described for the photomixotrophic *in vitro* culture of *Malus* cultivars (Damiano et al., 2002, 2003; Chakrabarty et al., 2005, 2007; Lucyszyn et al., 2006). This result may be explained by the applied PPFD (75 µmol m^{-2} s^{-1}) and will be discussed in the following. The removal of the vitamins resulted in a visible deterioration of the plant material especially on agar media (MS (+)/(-)), as can be seen in Fig. 5.18a and b as well as 5.18c and d (p. 88).

The results for the stomata analysis of 'Holsteiner Cox' leaves were obtained in the second and third run under 150 µmol m^{-2} s^{-1} in PA-TIS and the according reference cultures. The stomata of photoautotrophically cultivated plants showed a reaction to the dark period. As would be expected for fully functioning stomata, they were further closed

in darkness than under light (MS agar (-) and PA-TIS, Fig. 5.26, p. 96). The stomata of plants grown on MS agar (+), on the other hand, did not show any reaction and were even further opened in the dark than under light. The malfunction of stomata in photomixotrophic *Malus in vitro* cultures has been described by Brainerd and Fuchigami (1981, 1982) (through Zobayed et al. (2001a)). It has been attributed to the unusually high relative humidity and poor ventilation in closed micropropagation vessels. These observations accord with the findings in the present study. The influence of ventilation on the stomata will be discussed further below.

The best *Kalmia* shoot quality was observed in the PA-TIS. On agar, the influence of the abovementioned contamination of the plant material was very high and resulted in a poor plant quality and rooting efficiency (Fig. 5.34, p. 105). The influence of the different growth factors on the plant quality of both investigated plants will be presented hereafter. A stomata analysis on the abaxial leaf surface of *Kalmia* shoots could not be conducted since the leaves were too small to produce evaluable imprints.

6.3.1 Influence of carbon dioxide and ventilation on plant quality

When plants are flooded for a long time, such as in a continuous immersion culture, they are deprived of gases for photosynthesis and respiration. When the oxygen availability falls under 1 % O_2, it is essential for the plants to have access to sugars in order to survive by switching to anaerobic fermentation (Loreti et al., 2003). In the MS continuous immersion (+), the shoots were provided with sugar. The fresh and dry weight were much higher than in MS continuous immersion (-) (Tab. A.12, p. 185). As shown in Fig. 5.6b, the latter shoots were necrotic and had neither developed new leaves nor roots (Fig. 5.8a, p. 74 and Tab. 5.2, p. 75). According to Vartapetian and Jackson (1997), several plant injuries can occur under hypoxic conditions such as self-poisoning by products of fermentation (ethanol, lactic acid), cytoplasmic acidosis or energy starvation. Since the shoots in MS continuous immersion (-) had no means of reacting to the anoxic stress posed by submersion, for example by shoot elongation, these stress reactions led to the wilting of the plantlets. The 'Holsteiner Cox' shoots cultivated in MS continuous immersion (+), on the other hand, show typical reactions to oxygen shortage and high water availability such as prolonged and curved leaves (also described by Jackson et al. (1978)). They were able to produce a lot of fresh and dry weight even under poor aeration.

In the present study, the non-ventilated photomixotrophic culture on MS agar (+) grew significantly better than the likewise non-ventilated photoautotrophic one on MS agar without sugar. The leaves were larger in size and number (Fig. 5.8, p. 74), darker and not translucent as many of those on the sugar-free medium. The sugar in the medium compensated for the lack of carbon dioxide within the vessel. Morini and Melai (2003) showed that the addition of carbon dioxide to either photoautotrophic or photomixotrophic cultures of *Malus* rootstock MM106 resulted in higher fresh and dry weight than when the vessels were not ventilated. The highest dry matter content was achieved in the photomixotrophic culture (19 %). In the present study, more dry weight was produced in the continuous immersion culture of 'Holsteiner Cox' in MS (+) including vitamins than in any of the other treatments, even though the dry matter content was not the highest in this treatment (Tab. 5.2, p. 75). For *Kalmia*, only the fresh weight was determined, which was highest in the PA-TIS (Fig. 5.33, p. 105).

The difference between the dry weight accumulated in MS agar (-) and PA-TIS has to result from the carbon dioxide enrichment in the latter, because the medium composition did not differ except for the agar (8 g l^{-1}). Depending on the light intensity, the dry weight was 70 to 225 % higher in PA-TIS than on MS agar (-) (Tab. A.12, p. 185). With *Kalmia*, the fresh weight in PA-TIS was 84 % higher than on the corresponding agar culture (Tab. A.12, p. 185). Tisserat and Silman (2000) obtained equally high increases for the photoautotrophic cultures of oregano, peppermint and basil. They added either 1500, 3000, 10000 or 30000 μl CO_2 l^{-1} to a carbon dioxide chamber. The highest biomass accumulation was found under 10000 μl l^{-1} with a 25 times higher fresh weight than under non-enriched conditions (from approx. 0.2 g to 5 g) and a doubled multiplication rate. For a photoautotrophic *Eucalyptus* culture, the dry weight increase under 1200 µmol mol^{-1} was only 19 % when the plants were grown on a plastic net and 25 % on vermiculite as compared to non-enriched conditions (400 µmol mol^{-1}, Kozai and Kubota (2001)). Only when high exchange rates (2.5 h^{-1}) were paired with high PPFD (100 or 200 µmol m^{-2} s^{-1}) did the fresh and dry weight of banana shoots increase significantly (Nguyen et al., 2005). All of these results correspond to the observations on fresh and dry weight increase in the present study. In order to achieve such a biomass increase, the plants have to be photosynthetically active. Afreen et al. (2002b) showed that the dry weight of torpedo and precotyledonary stage embryos had too little chlorophyll and no stomata, so that a photoautotrophic cultivation was only possible from the cotyledonary stage on.

The 'Holsteiner Cox' shoots that were actively ventilated in PA-TIS and supplied with carbon dioxide showed fully functioning stomata in contrast to the plants on MS agar (+) (Fig. 5.26, p. 96). Additionally, significantly less and bigger stomata (Fig. 5.25, p. 96) were found on the leaves of plants grown in PA-TIS than either on MS agar (+) or (-). Zobayed et al. (2001a) detected the same trend for their study on ventilated *in vitro* cultures of cauliflower and tobacco. In airtight vessels, 1106 stomata per mm^2 were found on cauliflower and 961 per mm^2 for tobacco. The number decreased significantly when fast forced ventilation (10 cm^3 min^{-1} room air) was applied, lowering the number to 346 mm^{-2} and 309 mm^{-2}, respectively. The stomata in the air-tight vessels did not close during the dark period, either, whereas the ventilated cultures showed fully functioning stomata. In the present study, the mean number of stomata in PA-TIS was 125 $\pm$ 42, rising to 160 $\pm$ 58 in MS agar (-) and 187 $\pm$ 59 in MS agar (+). The difference was hence not as high as with Zobayed et al. (2001a), but comparable to the numbers found in the publication on *Limonium* by Lian et al. (2002): in the non-ventilated culture on medium containing sugar, 169 stomata per mm^2 were found whereas only 69 stomata per mm^2 developed under photoautotrophic conditions. In either publication, the lowered relative humidity in the vessels resulting from higher ventilation rates was made responsible for the low stomatal density and functionality of stomata closure.

The differing number between plants provided with carbon dioxide and those kept in close vessels may also be explained by the increased carbon dioxide concentration. Not only were less stomata found in the present study in the carbon dioxide enriched PA-TIS, but also in *in vitro* cultures of grape and coffee (Ross-Karstens et al., 1998). The more carbon dioxide was added to the vessels, the lower the stomatal density was. Woodward and Kelly (1995) tested 100 plant species on the relation of carbon dioxide and stomatal density. 74 % of the plants showed a significant decrease of stomata per mm^2 under elevated carbon dioxide concentrations.

It may be concluded that the stomatal density can reflect on the status of plants *in vitro*. Sufficiently ventilated plants develop less, but functional stomata than plants suffering from either too high relative humidity (see below) or too low carbon dioxide concentrations.

The carbon dioxide concentration in the PA-TIS was always maintained above 1200 µmol mol^{-1} independent of day or night phase. Teixeira da Silva et al. (2006) discovered in the photoautotrophic cultivation of *Spathiphyllum* that when carbon dioxide was only provided for 16 h, the fresh and dry weight of both roots and shoots was significantly lower than for a 24 h supply. No possible reasons were given for this ob-

servation, but nonetheless the carbon dioxide addition was not turned off in the present study during the night. Mostly, an addition of carbon dioxide was not necessary, because carbon dioxide was sufficiently produced by the plants.

Ventilation is imperative for the production of vital shoots. The addition of sugar to the medium may compensate for carbon dioxide deprivation in either immersion cultures or poorly ventilated vessels, but for photoautotrophic cultivation an enrichment of the vessel with carbon dioxide is essential. The efficient biomass production depends on the provision of the shoots with a carbon source as can be seen when comparing the continuous immersion or agar cultures without sugar addition to the PA-TIS treatments. A pairing with high light intensities (in this study at least 150 µmol m^{-2} s^{-1}) is necessary to fully exploit the photosynthetic potential of the explants.

6.3.2 Influence of lighting on plant quality

The influence of lighting on the plant quality was recorded in the first setup, when the light intensity was increased from 115 + 75 to 115 + 100 µmol m^{-2} s^{-1}, and in the second setup, when the lighting cycle was changed from 16/8 h light/dark to 24 h continuous.

The increase of light intensity in PA-TIS in the first setup resulted in an increase of the average fresh weight by 26 % and of the dry weight by 22 % (Tab. A.12, p. 185). Kubota et al. (2001) also detected an increase in the dry weight of photomixotrophically cultivated tomato plantlets under higher PPFD: the dry weight changed from 105 to 178 mg when the PPFD was increased from 70 to 100 µmol m^{-2} s^{-1}. In the photoautotrophic cultivation, however, the dry weight decreased from 107 mg to 88 mg. This does not correspond to the results of the present study. No significant increase was detected when peppermint and thyme were cultivated on sugar-free medium even though the light intensity was increased from 80 to 180 µmol m^{-2} s^{-1}. In both studies, the vessels were not enriched with carbon dioxide, so that the high light intensity could not be used for a higher photosynthetic activity. Nguyen et al. (1999a), on the other hand, varied both carbon dioxide and PPFD in the photoautotrophic cultivation of *Coffea arabusta*. When the PPFD was increased from 150 to 350 µmol m^{-2} s^{-1} under non-enriched carbon dioxide conditions, the difference between the dry mass under the lower and higher light intensity was not significant (41.0 mg to 61.8 mg). The impact of increasing the carbon dioxide concentration (without changing the PPFD) was much higher (41.0 mg to 132.1 mg). Increasing both PPFD and carbon dioxide supply did, surprisingly, not result in more dry

mass, but less than before (though not significantly less).

An influence of the simultaneously higher vessel and room temperature (Fig. 5.5, p. 68) on the increase detected in the present study cannot be excluded, since the fresh weight of the shoots on MS agar (+) was also higher (increase from 680 to 784 mg) although the PPFD in the reference cultures was not increased. Cui et al. (2000) showed in the photomixotrophic cultivation of *Rehmannia glutinosa* that both temperature and PPFD had a significant influence on the plant height and biomass accumulation. A significant interaction of the two growth factors was described for fresh and dry weight. The average leaf length in the present study was higher under 115 + 100 µmol m^{-2} s^{-1} in PA-TIS (3.3 cm) than under 115 + 75 µmol m^{-2} s^{-1} (2.9 cm). But the leaf length of the reference cultures likewise increased between the treatments (see Fig. 5.8, p. 74): from 3.1 to 3.4 cm for MS agar (+) and from 2.1 to 2.6 cm for MS agar (-). Therefore, the influence of other factors besides lighting on the plant quality cannot be excluded for the first setup.

In the second setup, the duration of the light period was increased to 24 h. Roitsch and Sinha (2002) suggested continuous lighting in a photoautotrophic culture in order to accumulate more chlorophyll. The fresh and dry weight in all runs under discontinuous lighting (16/8 h) was lower than under continuous lighting (Tab. A.12, p. 185). The number of leaves (Fig. 5.20, p. 92) increased for all treatments with the 24 h-lighting. A comparison to the first setup concerning the influence of light is not possible since the changing of the medium composition had a strong impact on plant growth. The prolongation had a significant influence on the rooting efficiency in the second setup and will be discussed in the following.

The stomatal density of *in vitro* plants may not only depend on the ventilation (see above) or relative humidity (as will be discussed below), but also on the lighting conditions. In the present study, 'Holsteiner Cox' shoots were cultivated under a PPFD of 150 µmol m^{-2} s^{-1} in PA-TIS as compared to 75 µmol m^{-2} s^{-1} in the reference cultures. Kim et al. (2004) showed that more stomata were formed by chrysanthemum plantlets under LED combination of blue and far-red (98.7 mm^{-2}) than under fluorescent lamps (76.9 mm^{-2}). The PPFD was regulated to 50 µmol m^{-2} s^{-1}, so that the influence of different light intensities was excluded. The plants under the LEDs also developed significantly less fresh weight (255 mg) than under fluorescent lighting (713 mg), so that it can be assumed that this combination was not beneficial for the plants' growth. Hence, the lower number of stomata also may have been influenced by the high light intensity applied to PA-TIS.

For *Kalmia*, the PPFD was not changed in between the runs, the reference cultures were kept under 75 µmol m^{-2} s^{-1} whereas the plants in PA-TIS were cultivated under 150 µmol m^{-2} s^{-1}. The plant quality in PA-TIS was much better than in the agar treatments which may partly be attributed to the higher light intensities. In Chapter 6.3.3, the anthocyanin-production of the plants on WPM agar (+) as a reaction to both lighting and sugar-addition will be discussed.

6.3.3 Influence of medium and supporting material on plant quality

The contact of *in vitro* shoots with the cultivation medium ensures a steady supply of nutrients. The problems of a continuous immersion within this medium are not only related to the aforementioned oxygen deprivation, but also to a high availability of water. These may lead to the following morphological and physiological disorders described by Ziv (2000): translucent, waterlogged tissue, disorganized growth of the shoot system, thickened stems, and curled, abnormal leaves. The shoot depicted in Fig. 5.6a shows most of these symptoms. The leaves in PA-TIS were longer than in the liquid culture (Fig. 5.8), different to the observations by Escalona et al. (1999) for the cultivation of pineapple, where the leaves in TIS were smaller. The plants grown in MS continuous immersion without sucrose showed also signs of stress. The coloring of the medium (Fig. A.1, p. 173) indicates to a phenolic excretion, a stress reaction. The application of temporary immersion improved the quality of the shoots significantly, although even in PA-TIS hyperhydricity occasionally occurred (Fig. A.2b and c).

This phenomenon could be overcome by shortening the immersion times to less than 6 minutes. In the present study, the immersion cycles were not varied in order to keep the influence of the flooding constant. Damiano et al. (2003) measured both the highest and the lowest dry matter content of *Malus* cultivar 'Jork 9' in TIS, depending on the duration of immersion. When the plants were submitted to 30 minutes of immersion per day, the dry matter content was very low (2.4 %), whereas it was very high when the immersion was prolonged to 60 minutes (21.5 %). The percentage of hyperhydric shoots was not presented, but it was mentioned that hyperhydricity could be reduced by the variation of immersion. This may be the grounds for future studies on PA-TIS.

The dry matter content in the MS continuous immersion (+) culture (10.3 ± 1.3 %) was comparable to the culture on MS agar (+) (10.3 ± 1.5 %) (Tab. A.12, p. 185). For the cultivation of the *Malus* rootstock 'M9 EMLA', the fresh and dry weight relations were

comparable. The fresh weight was higher under continuous immersion (241 mg) than in TIS (207 mg), as it was also recorded in the present study (871 mg vs. 480 mg). The dry matter content of 'M9 EMLA' in TIS (10.5 %) was also higher than under continuous immersion (6.8 %) (HC: 10.3 % in continuous immersion, 11.5 % in TIS). Interestingly, quite different results were obtained for other *Malus* scion cultivations: Damiano et al. (2002) only recorded a DMC of 7.7 % for the continuous immersion culture of 'Gala' as compared to 7.1 % on solid medium and 6.9 % in TIS. The dry matter content in continuous immersion was hence higher than in TIS, contrary to the results found here. The DMC seems to strongly depend on the provision of the shoots with medium and its composition. Dry matter contents as low as 8.6 % were found in MS continuous immersion (-). In the present study, the DMC of the least successful treatment was still higher than in the publication by Damiano et al. (2002). This indicates to a strong influence of the *Malus* cultivar chosen on the results in temporary immersion.

As already mentioned above, the addition of sugar to the continuous immersion culture resulted in accumulation of dry weight of the plantlets whereas those deprived of an exogenic carbon source wilted (Fig. 5.6c). Not only did the photomixotrophic plants in the liquid culture produce more dry weight than the photoautotrophically cultivated shoots, but also on MS agar (+). The photomixotrophic treatment proved to be beneficial for the plant quality and biomass production of either 'Holsteiner Cox' leaves or roots, even though no ventilation was applied. Only when no vitamins were added to the medium did the shoots in the PA-TIS accumulate more biomass than the corresponding photomixotrophic culture on MS agar (+) and the non-ventilated photoautotrophic culture on MS agar (-). Morini and Melai (2003) also detected a higher biomass accumulation in the photomixotrophic culture of MM106 as compared to the photoautotrophic one. Independent of the carbon dioxide addition, fresh and dry weight were higher when sugar was present. For *Malus* × *domestica* cv. 'Gala', de Medeiros Rodrigues et al. (2006) obtained the best multiplication rate for both internodal segments and stem apices on MS agar supplied with 30 g l^{-1} and the worst for 0 g l^{-1}. The same observations were made by Rodríguez et al. (2003). The results of the present study for *Malus* are thus consistent with literature.

The cultivation of *Kalmia* in PA-TIS, however, was more successful. The fresh weight and shoot length were almost doubled in PA-TIS as compared to the agar reference cultures (Fig. 5.33, p. 105). Interestingly, the fresh weight and stem length on agar did not differ significantly for WPM (+) or WPM (-), although the shoots were not additionally ventilated in the vessel without sugar. This result is thus different from what was detected

for 'Holsteiner Cox'. The reasons for this may be found in the plants' morphology: On WPM agar (+), the stems and leaves were red as can be seen in Fig. 5.35a, p. 106, which indicates to a stress. Chawla (2002) summarizes the possible reasons for reddening of plant stems *in vitro*: too much sugar, too little nitrate or too old cultures. It is possible that the sucrose concentration of 20 g l^{-1} in the medium was too high for *Kalmia* since the plants on WPM (-) were completely green and plants in PA-TIS were only partially red. Riechers (1993) also detected a reddening in the cultivation of the *Ericaceae Vaccinium vitis-idaea* 'Red Pearl' with sucrose concentrations as low as 10 g l^{-1} The red color was attributed to a high anthocyanin production. Anthocyanins are antioxidant pigments produced for the protection of the plant from light damage by absorbing excess radiation (McDonald, 2003, pp. 27 - 29). Since the *Kalmia* shoots on WPM agar (+) were provided with an exogenic carbon source and did not depend on photosynthesis, the PPFD of 75 µmol m^{-2} s^{-1} might have been too high. The photoautotrophic plants, on the other hand, had green leaves and were photosynthetically active. Additionally, the bacterial contamination on WPM agar (+) was higher than on WPM agar (-) and not apparent in PA-TIS (Fig. 5.34). This infection might have led to an additional decrease in the plants' quality. The bacteria were deprived of sucrose in both photoautotrophic treatments. Interestingly, on WPM agar (-) a bacterial film formed around the *Kalmia* shoots. Apparently, the plants excreted carbohydrates into the medium which provided a breeding ground for the bacteria. This may be an interesting aspect for future studies on whether photoautotrophic cultures really are less prone to contaminations. In the cultivation of *Kalmia*, vitamins were added to the medium; hence it was not completely free of exogenic carbon.

Since Kubota (2001) and Sha Valli Khan et al. (2003) defined an actual photoautotrophic culture to be free of any exogenic carbon source, the vitamins were removed from the medium composition in the cultivation of 'Holsteiner Cox' in the second setup. Only under these conditions did the shoots in PA-TIS grow better and produce more fresh and dry weight than the photomixotrophic control (Fig. 5.19, p. 91). The Murashige and Skoog (1962) vitamin mixture contains glycine, myo-inositol, nicotinic acid (vitamin B_3), pyridoxine (vitamin B_6) and thiamine (vitamin B_1). Linsmaier and Skoog (1965) revised all these vitamins and ascertained that thiamine is essential for the growth of *in vitro* plants. Consequently, the removal from the medium resulted in a much lower quality and rooting efficiency of the 'Holsteiner Cox' control cultures as well as the plants in PA-TIS. Since the removal of vitamins from the medium composition had such a negative influence on the plant growth, the *Kalmia* cultures were conducted with vitamins again (Tab. 4.4, p. 40).

The loss of plants due to contamination was higher in the vessels containing agar medium than in PA-TIS. No contaminations were detected in the photoautotrophic culture of 'Holsteiner Cox', whereas fungal infections (Fig. A.1b, p. 173) occurred when sugar was present. These plants were not evaluated for fresh or dry weight, since the fungal infection had an effect on growth due to the competition for nutrients. Of all 104 vessels containing 'Holsteiner Cox' on MS agar (+), 6 were contaminated (6 %). All vessels containing *Kalmia* shoots on agar medium, independent of the addition of sugar, were contaminated (100 %). In commercial plant production, contaminated vessels would not have been used anymore, because fungal and bacterial infections pose a threat on plant health and productivity. Winkelmann et al. (2006) report problems in the commercial production of *Kalmia* due to endophytes and rooting difficulties. In PA-TIS, plants were not lost due to contamination but to malfunctioning of the pumps (Fig. A.3, p. 174). One of 26 vessels (both HC and KL, 4 %) was hence not available for further studies. As opposed to the contamination, this loss could have been prevented by adding a moisture sensor in height of the plants into the PA-TIS.

The nutritional status of the plants had an influence on the functionality of the stomata (closure in the dark period) as well as the stomatal size and density. The difference between the plants on MS agar with or without sugar cannot result from differing ventilation or relative humidity, since only the sugar concentration was varied. The stomata opening on MS agar (-) was significantly lower during the dark period than in the light (decrease from 60 % to 48 %). More stomata opened 51 to 75 % where found in the dark period on MS agar (+) than in the light, resulting in a higher percentage of opened stomata in darkness (dark: 31 %; light: 38 %; see Fig. 5.26 and 5.27, pp. 96 - 97). The dependence of the photoautotrophic plants under non-enriched conditions (MS agar (-)) on carbon dioxide instead of sugar as a carbon source must have resulted in a significantly higher opening of the stomata in the light (60 %) as compared to the photomixotrophic treatment (31 %). The number of stomata also differed significantly between both cultures ((+): 186 mm^{-2}; (-): 160 mm^{-2}). A corresponding trend was found by Jo et al. (2008) in the cultivation of *Alocasia amazonica* on different sugar concentrations. Without sugar, 43 stomata per mm^2 were developed instead of 84 mm^{-2} on medium containing 3 g l^{-1} sucrose or 98 mm^{-2} for 6 g l^{-1}. This increase was also detected by Lian et al. (2002), where a ventilated photoautotrophic *Limonium* culture was compared to an equally ventilated photomixotrophic culture. Under photoautotrophic conditions 69 stomata mm^{-2} were developed in comparison to 76 mm^{-2} under photomixotrophic conditions. Here, both numbers were relatively low due to the provision with carbon dioxide enriched air (1500 µmol mol^{-1}), but still the sugar addition led to a stomatal density increase. The

stomatal density of *Calathea orbifolia* was calculated in a TIS and reference culture on semi-solid medium by Yang and Yeh (2008). In TIS, the stomatal density was lower (36.8 ± 0.6 mm^{-2}) than in the agar culture (48.5 ± 1.9 mm^{-2}). The reason given was the better ventilation in TIS than in the conventional vessel, because the headspace was exchanged with every immersion cycle. In the present study, the lowest number of stomata per mm^2 was also found in the temporary immersion treatment.

6.3.4 Influence of relative humidity on plant quality

The variation of relative humidity between the treatments under the same lighting condition in PA-TIS was not high (Tab. A.10, p. 184). Like in most publications on ventilated photoautotrophic cultures (see Tab. A.3, p. 178), the relative humidity lay under thenormally assumed rH of 100 % *in vitro* (Zobayed et al., 1999c, 2002). Physical abnormalities of *in vitro* cultivated shoots are normally attributed to the high relative humidity which leads to problems in the plants' transpiration (Vanderschaege and Debergh, 1987). For both 'Holsteiner Cox' and *Kalmia latifolia*, no hyperhydricity was detected in the agar cultures. In PA-TIS, on the other hand, some shoots showed signs of hyperhydricity (Fig. A.2) even though the relative humidity was low. This may be attributed to the functionality of temporary immersion. It is assumed that the medium stays as a film on the shoots after the flooding, so that nutrient transport is possible but gaseous exchange is not limited (Damiano et al., 2003). In this case, the occurrence of deformed shoots may not be attributed to high relative humidity but to overprovision with medium. As mentioned above, a variation of the immersion cycles for the 'Holsteiner Cox' shoots should be considered. The *Kalmia* shoots did not suffer from deformations and the overall performance in PA-TIS was much better than on agar.

The relative humidity has been described to have an immediate effect on the development and functionality of stomata *in vitro* (Zobayed et al., 2001a; Lian et al., 2002). By increasing ventilation or not closing the vessel completely (vents, filters), it can be reduced significantly (Seelye et al., 2003). In the present study, both ventilation and bottom cooling lead to low relative humidity. As mentioned above, the stomata in PA-TIS and on MS agar (-) showed a normal functionality with closure in the dark period. The relative humidity was only determined in PA-TIS (< 90 %; Tab. A.10, p.184) and supposedly much lower than in the reference culture (up to 100 % according to Zobayed et al. (1999c, 2002)). Hence it is no surprise that the opening on MS agar was much higher ((+): 38 %; (-): 48 %) than in PA-TIS (28 %; Fig. 5.26, p. 96). For *Rehmannia glutinosa*, Seon et al.

(2000) described that high respiration rates in photomixotrophic cultures (glycolysis) lead to stomata malfunction. The need for medium uptake is elevated by the resulting water loss through the opened stomata. Thereby, potassium, which is responsible for stomata regulation, is taken up in excessive amounts. The stomata are overregulated and cannot close anymore, leading to even higher respiration rates. In photoautotrophic cultures, the respiration is not high and hence the stomata can close properly.

Chakrabarty et al. (2005) named high relative humidity one of the most important elicitors for hyperhydricity in tissue cultures. They showed the immediate effect of hyperhydricity on the stomata of *Malus* rootstock 'M9 EMLA'. Deformed stomata such as presented in the micrographs of hyperhydric leaves were not detected in any of the treatments in the present study which shows that the bottom cooling treatment and lowering of the relative humidity in fact was beneficial for the plants in PA-TIS.

Egbers (2005) discussed the negative influence of bottom cooling on *Phalaenopsis* cultures on agar. This was attributed to the low temperature of the medium. The same effect could not be detected in the present study, since the plants, being separated from the fluid through the carrier when not flooded, were not in contact with the cooling all the time. Additionally, the temperature was lowered which proved to be beneficial for the net photosynthetic rate (see Chapter 6.2.5). For future studies in temporary immersion systems, the application of bottom cooling is hence recommendable. The effect of temperature on the plant quality will be discussed hereafter.

6.3.5 Influence of temperature on plant quality

The effect of temperature in the first setup on the plant quality of 'Holsteiner Cox' in PA-TIS is not easily discernible, since the temperature increase of 2 K between the second and the third run shown in Fig. 5.5, p. 68 was unintentional and emerged simultaneously with the change of PPFD from 115 + 75 to 115 + 100 µmol m^{-2} s^{-1}. The reference cultures, on the other hand, were kept under the same PPFD and can therefore be used for the discussion. As mentioned above, the fresh and dry weight of shoots grown on MS agar (+) increased when the temperature rose (Tab. A.12, p. 185). In the second setup, the same trend was detected for the plants on MS agar (+) without vitamins. As can be seen in Fig. 5.19a (p. 91), the fresh mass differed significantly between the first and second run. This decrease in fresh weight occurred when the temperature was lowered from almost 26 °C to 24 °C. Interestingly, this effect is, again, only visible for the

photomixotrophic culture. In the photomixotrophic liquid culture of *Alocasia amazonica* (30 g l^{-1} sucrose added) under different temperatures (20, 25, 30, 35 °C) a similar effect of the temperature on the culture was detected: at 25 °C, the number of leaves and fresh weight per shoot was highest. Lower and higher temperatures led to a decrease of both leaf number and fresh weight (Jo et al., 2008).

In the photoautotrophic culture in PA-TIS, the effect of the temperature was opposite. Significantly more fresh weight was produced under lower temperatures (476 mg), but also less leaves (Fig. 5.19a and 5.20a). The reason for the different reactions in the agar and PA-TIS cultures may lie in the assumption that the temperature in the vessels was comparable. The temperature in PA-TIS was influenced by the different factors discussed in Chapter 6.1.5, but especially the bottom cooling. The vessels containing agar, on the other hand, were not provided with a separate cooling system and not influenced by either pumps or valves heating up the cultivation air. The room temperature was measured in between the vessels with a NTC. Even though the temperature within the vessels is not known, the room temperature and the difference to the reference cultures' temperature may provide a ground for discussion. The temperature in the third run under 115 + 100 µmol m^{-2} s^{-1} was, for instance, 3 K higher in PA-TIS than in the room (Tab. A.11, 184). Similarly, the room temperature was much lower in the third run under 150 µmol m^{-2} s^{-1} (again by 3 K). By providing the vessels containing agar medium with light, the temperature might have increased by 1 to 2 K as suggested by Kubota et al. (1997) and Zobayed et al. (2001a). Nonetheless, it would have been lower than in the PA-TIS. The differences between day and night temperatures were hence even more extreme in the agar vessels, resulting in a positive DIF of up to 6 K (see Tab. A.11, p. 184). This can also be seen in Tab. A.11, where all temperatures are listed for the different treatments. Cui et al. (2000) stated for the cultivation of *Rehmannia glutinosa* that positive DIF are beneficial for plant growth and biomass accumulation. Changing the temperature from 25/25 °C day/night to 26/18 °C resulted in a significantly higher fresh and dry weight under 210 µmol m^{-2} s^{-1} (36 and 41 % more, respectively).

The difference between the MS agar (+) and PA-TIS treatments can thus partly be attributed to the involuntary DIF-treatments the plants on agar were submitted to. For future studies, a lowering of the temperature in PA-TIS during the night might be a means of increasing the biomass production.

6.4 Rooting and acclimatization success

The rooting efficiency of 'Holsteiner Cox' shoots on agar *in vitro* was, as already mentioned, satisfactory to begin with. The shoots were chosen for the establishment of PA-TIS because rooting protocols for agar cultures were available (Szankowski, 2002). Also, *Malus* shoots had already been cultivated successfully under both photoautotrophic conditions (Morini and Melai, 2003) and temporary immersion (Damiano et al., 2002, 2003; Zhu et al., 2002, 2005). Rooting of *Malus* had already been shown to work for M26 rootstock in RITA® vessels (Zhu et al., 2005), but had not yet been described for photoautotrophic cultures. The rooting efficiency in the present study varied strongly in the different treatments: no roots were formed at all in the reference liquid cultures, 80 to 100 % of the shoots rooted on MS agar (+), 7 to 41 % on MS agar (-), and finally 38 to 96 % in PA-TIS. The rooting strongly depended on the medium composition and lighting conditions the 'Holsteiner Cox' shoots were submitted to.

The rooting of *Kalmia latifolia* 'Ostbo Red' had proven to be problematic in preliminary tests at the Tree Nursery Section (Leibniz Universität Hannover). The low rooting efficiency on WPM agar containing sucrose was confirmed in the present study, with only 23 % rooted shoots (Tab. 5.8, p. 106). Surprisingly, *Kalmia* reacted positively to the rooting medium without sucrose and rooted at 60 %. In PA-TIS, the highest efficiency was found (on average 89 %). In Fig. 5.36 (p. 107) the results for root length are summarized; roots in PA-TIS were significantly longer than on WPM agar (-) and comparable to those on MS agar (+). Since only rooted shoots were included in this Figure, the actually poor quality of the shoots on WPM agar (+) is not really reflected.

The acclimatization success of *Kalmia latifolia* can mainly be attributed to the high rooting percentage in PA-TIS. Debergh and Maene (1981), Kataoka (1994) and Kozai and Zobayed (2000) name the low rooting efficiency of *in vitro* grown plants as well as malformed roots and thereby resulting problems in water uptake to be the main causes of poor acclimatization success. Roots developed *in vitro* are believed to compensate for water loss after the *ex vitro* transfer caused by malfunctioning stomata (Seelye et al., 2003). Accordingly, the survival rate of apple microcuttings rooted *ex vitro* has been shown to be lower than of *in vitro* rooted microcuttings by De Klerk (2000). After the rooting in photoautotrophic systems, higher survival rates have been reported by Aitken-Christie et al. (1995), Xiao and Kozai (2004), and Zobayed et al. (2004a) for photoautotrophic plantlets than for plants cultivated conventionally.

Of the *Kalmia* shoots rooted in PA-TIS, 85 % were successfully established on peat substrate (Tab. 5.9, p. 107). The rooting intensity played an important role in that. As can be seen in Fig. 5.40 (p. 109), the strongly rooted plants (5.40a) grew more vigorously than those without roots (5.40c). The percentage of plants lost in the hardening process was especially high in non-rooted plants coming from the photomixotrophic treatment on WPM agar (+) (Fig. 5.38b). Of 110 weakly or not rooted shoots, only 62 were acclimatized after 4 weeks (56 %, Fig. 5.37). One of the reasons therefore might have been the stronger development of callus on WPM agar (+) than in the other cultures. Nguyen et al. (1999a) suggested that callus in the rootzone lowers the chance of survival *ex vitro*. The working stomatal closure detected for photoautotrophic 'Holsteiner Cox' plantlets either in PA-TIS or WPM agar (-) may have likewise led to lower loss rates than for those on WPM agar (+) (stomatal density and opening not determined for *Kalmia*).

In the following, the influence of the different growth factors on the rooting and survival *ex vitro* will be discussed.

6.4.1 Influence of carbon dioxide and ventilation on rooting and acclimatization success

The overall better rooting of *Malus* or *Kalmia* in PA-TIS than on MS or WPM agar (-) (without sugar, PA non-enriched) may be attributed to a large extent to the ventilation and enrichment of PA-TIS with carbon dioxide. When vitamins were added to MS agar (-), up to 40 % of the 'Holsteiner Cox' shoots rooted (Tab. 5.2, p. 75). At the same time, more roots were produced in PA-TIS (53 - 73 %). Even more shoots rooted when the lighting was increased (up to 96 %). For *Kalmia*, the rooting percentage was not only better in PA-TIS (89 %) than for the control on WPM agar (-) (60 %), but also for WPM agar (+) (23 %). Tisserat and Silman (2000) also detected an increase of rooting by oregano, peppermint and basil when high carbon dioxide concentrations were provided (10000 μl CO_2 l^{-1}). For both photoautotrophic or photomixotrophic cultures, significantly more roots were formed when carbon dioxide enrichment took place. Other publications even report higher rooting successes in photoautotrophic than in conventional cultures. 100 % rooted shoots were found in the photoautotrophic culture of St. John's Wort whereas the efficiency of the photomixotrophic culture was only 73 % (Couceiro et al., 2006). The root fresh and dry weight were significantly higher under photoautotrophic conditions. Zobayed et al. (2000b) also detected higher root dry and fresh mass in the photoautotrophic treatment for *Eucalyptus* than in the photomixotrophic

control (168 to 127 mg and 20 to 14 mg, respectively). The highest percentage of rooted coffee plants was found by Afreen et al. (2002b) in their self-built TRI-bioreactor (90 %). Under temporary immersion in the commercially available RITA®, on the other hand, an even lower rooting capacity (29 %) was detected than in the photomixotrophic control (57 %). Again, the fresh and dry weight of the roots was significantly higher under photoautotrophic conditions.

In the present study, the fresh and dry weight were not determined separately for the roots. Only the number and length of the roots were measured. The root number was not determined for *Kalmia*, but only the rooting intensity. It was divided into strong (more than 10 roots), weak (less than 10 roots) and non-rooted. Under carbon dioxide enrichment, significantly longer roots were formed by 'Holsteiner Cox' in PA-TIS than on MS agar (-) when vitamins were added to the medium (Fig. 5.10d, p. 77) and a significantly higher number of longer roots was detected under high light intensities in PA-TIS (150 µmol m^{-2} s^{-1}, both 16/8 h or 24h lighting) when no vitamins were added (Fig. 5.28, p. 98). In the rooting of *Kalmia*, more strongly rooted plantlets were found in PA-TIS than in the agar treatments (Tab. 5.8, p. 106). Similar results were obtained in the photoautotrophic cultivation of *Eucalyptus* (Tanaka et al., 2005). The higher the carbon dioxide enrichment was, the more roots were formed. No roots were found in the control, whereas under a carbon dioxide concentration of 2000 or 3000 µmol mol^{-1}, 3.2 roots per shoot were counted.

Not only did the supply with carbon dioxide have a positive effect on the rooting, but also on the acclimatization success. Of the *Kalmia* shoots provided with carbon dioxide, 85 % could be established *ex vitro*, of those in the non-ventilated treatments, only 62 % and 78 % survived coming from WPM agar (+) and (-), respectively. Even lower survival rates were detected by Xiao and Kozai (2004) for calla lily. The survival was evaluated after 12 days. 60 % of the photomixotrophically cultivated plantlets had been established whereas 95 % of the photoautotrophic plants had survived. Seon et al. (2000) reported almost identical acclimatization rates for *Rehmannia glutinosa ex vitro* (PM: 64 %, PA 92 %) which is similar to the rates determined for *Kalmia* (PM: 62 %, PA: 85 %). One of the possible reasons for the higher survival by photoautotrophically grown plants was suggested by Kirdmanee et al. (1995a). Since the plants already apply photosynthesis *in vitro*, they are already 'hardened' *in vitro* during the rooting phase. This statement is also reinforced by the results of Makunga et al. (2006). When *Thapsia garganica* L. shoots were ventilated during the rooting stage, 75 % of the rooted shoots survived as compared to 35 % of the non-vented shoots. Under both conditions, the non-rooted plants did not

survive *ex vitro*.

One of the most important influences of ventilation on the plant quality and rooting efficiency, the lowering of the relative humidity, will be discussed in Chapter 6.4.4.

6.4.2 Influence of lighting on rooting and acclimatization success

In the present study, the lighting was only varied in PA-TIS for the cultivation of 'Holsteiner Cox'. When the lighting was increased from 115 + 75 µmol m^{-2} s^{-1} to 115 + 100 µmol m^{-2} s^{-1}, the average number of roots increased at the same time. The root length did not increase significantly, since it was above average in the second run under 115 + 75 µmol m^{-2} s^{-1} (Fig. 5.10, p. 77). Aragón et al. (2010) detected in the photomixotrophic cultivation of plantain much longer roots (15 cm) under low light conditions (80 µmol m^{-2} s^{-1}) with high carbon dioxide application (1200 µmol mol^{-1}) than under high light (150 µmol m^{-2} s^{-1}, 11.4 cm), which would correspond to the high root length found in the second run under 115 + 75 µmol m^{-2} s^{-1}.

The increase of PPFD to 150 µmol m^{-2} s^{-1} can, unfortunately, not be compared to those values, because the influence of removing the vitamins from the medium composition was higher than the influence of a higher PPFD. The duration of the light period was changed in the second setup from 16/8 h light/dark period to continuous lighting. This resulted in overall more and longer roots (Fig. 5.28, p. 98) and a higher rooting success (increase from 72-81 % to 84-96 %). This is an interesting result, since oftentimes rooting is initialized by incubation in the dark. Zhu et al. (2002, 2005), for instance, submitted the apple rootstock M26 to a treatment with IBA-containing medium (1.2 µM) in complete darkness for four days, and later cultivated the plants without IBA. The rooting success lay between 91 and 100 %, depending on pretreatment in the multiplication and elongation phase. Druart et al. (1982) also reported that the application of continuous darkness induced higher percentages of rooted plantlets. Thereby, a maximum of 4.4 roots per Jonagold shoot were obtained, a much lower number than here under continuous lighting (on average 7 - 9 roots per rooted 'Holsteiner Cox' shoot, Tab. A.13, p. 186).

The high rooting success (60 %) of *Kalmia* on WPM agar (-) may be explained by the transparent vessels they were cultivated in in the present study. In the Tree Nursery Section (Leibniz Universität Hannover), the rooting efficiency was low (under 25 %). There, *Kalmia* are micropropagated and rooted in glass jars with opaque twist-off lids. Oftentimes, the jars are even stacked for space saving. Under these conditions, the applied

PPFD will be much lower in the vessels than in the present study, where 75 µmol m^{-2} s^{-1} were used. For future investigations on *Kalmia* rooting, the increase of the light intensity might be considered because of the overall higher rooting efficiency under high PPFD in PA-TIS.

6.4.3 Influence of medium and supporting material on rooting and acclimatization success

The differences in rooting between the treatments of either 'Holsteiner Cox' or *Kalmia* shoots on agar (+) or (-) has to derive from the sucrose concentration, since no other parameters were changed. The 'Holsteiner Cox' rooting on MS agar (+) was overall more successful than on MS agar (-) (Tab. 5.2 and 5.6, pp. 75 and 99). The number of roots (Fig. 5.10/5.28 a+c) and root length (Fig. 5.10/5.28 b+d) was significantly higher in any of the treatments, independent of vitamin removal or prolongation of the light period. Jo et al. (2008) discussed the influence of sugar on the rooting of *Alocasia amazonica* and also found that without sucrose, fewer roots (6.3 per plant) were formed than with either 30 g l^{-1} (9 roots per plant) or 60 g l^{-1} (10.3 roots per plant). Since the plants on MS agar (-) were not ventilated and hence suffered from carbon deprivation (no CO_2, no sugar), this result is not surprising. The plants in PA-TIS, which were ventilated, fared much better than the ones on MS agar (-) (see Chapter 6.4.2).

The *Kalmia* shoots on sugar-free medium, on the other hand, reacted much better than the ones on WPM agar (+), contrary to the observations for 'Holsteiner Cox'. The strong bacterial contamination in the photomixotrophic treatment may have played an important part in this result (see Fig. 5.34, p. 105). The use of only the shoot tips for the multiplication step suggested by Riechers (1993) did not lead to an elimination of these endophytes. Although some bacteria grew on WPM agar (-) close to the plants (a possible excretion of carbohydrates as breeding ground was already named above), the bacterial growth rate was much lower than with sugar present. In PA-TIS, where the highest rooting efficiency (89 %) was detected, no clouding of the medium due to bacterial contamination was recorded. The relatively short contact of the medium with the plant material might thus have played an important part in preventing bacteria from limiting the root formation and plant growth.

For the induction of roots, plant growth regulators were added to the medium. The

IBA-concentration for the 'Holsteiner Cox' rooting had been tested and optimized by Szankowski (2002), so that the rooting efficiency was high (up to 100 %) in the MS agar (+) control. The superiority of IBA over other auxins for the rooting of *Malus* was already described by van der Krieken et al. (1993). When Morini and Perrone (2006) omitted IBA from the medium composition, MM106 developed no roots under a 16/8 h light/dark cycle, but up to 4 roots per shoot were formed with 0.4 mg l^{-1} IBA. Another means of increasing the root number *in vitro* was described by Zobayed et al. (1999b, 2001b). The addition of ethylene perception inhibitors such as $AgNO_3$ and ACC led to a higher number and longer roots for cauliflower and potato. This may be the grounds for future studies on PA-TIS, since ethylene concentrations were not yet determined.

The substrate, on which plants are rooted *in vitro*, has proven to play an important part on the rooting success. In the continuous immersion culture, no roots were formed in the present study (Tab. 5.2, p. 75). The roots in agar and in PA-TIS differed in their physiology: callus-formation was detected in agar for both 'Holsteiner Cox' (Fig. 5.18b, p. 88) and *Kalmia* (Fig. 5.35b, p. 106), whereas the roots developed directly from the lowest internodes in PA-TIS (Fig. 5.6f, 5.18e+f and 5.35c). The roots in agar were thinner than in PA-TIS. For *Kalmia*, in contrast to the results for 'Holsteiner Cox', the roots were more numerous in PA-TIS than on agar (higher number of strongly rooted shoots, see Tab. 5.8).

The use of porous substrate in photoautotrophic cultivation to improve the root quality and the later transferability *ex vitro* was suggested by Kubota and Kozai (1992) (rock wool), Xiao et al. (2000) (perlite), Heo and Kozai (1999) (vermiculite and cellulose plugs) or Afreen et al. (1999) (florialite). Porous substrates improve the provision of the roots with air, which usually is limited in solidified media by the use of agar or gelrite. For the rooting of M9 rootstock, the efficiency on agar was compared to vermiculite or vegetary ash as substrate (Vieira et al., 2007). The highest number of rooted shoots was detected on vermiculite (88.4 %) followed by vegetary ash (87.9 %) and agar (79.1 %). Contrary results were obtained by Pasqual et al. (2000) for M7, where no rooting was detected on vermiculite, but on agar in different concentrations (3 or 6 g l^{-1}). A substitution of agar for the galactomannan guar was suggested by Lucyszyn et al. (2006), because the cultivar 'Marubakaido' rooted best when a part of the agar was substituted for guar. In the present study, the use of no substrate at all in PA-TIS did not limit the rooting efficiency, because up to 96 % of the 'Holsteiner Cox' and 89 % of the *Kalmia* shoots rooted. Since only temporary immersion was provided, the roots did not suffer from undersupply of aeration. In the photoautotrophic cultivation of *Pleioblastus pygmea* more roots were

formed without supporting material than with vermiculite, which shows that the omission of carrier substances may be beneficial for the rooting (Watanabe et al., 2000).

Additionally, it makes the *ex vitro* transfer much easier when no carrier substance has to be removed from the shoots, because this usually leads to injury of the roots. Agar and especially sugar residues have to be removed from the shoots before the transfer to soil, so that a cleaning step has to be integrated for these shoots (Roberts and Smith, 1990; Heo and Kozai, 1999). In PA-TIS, only MS or WPM salts were used, so that the contamination risk *ex vitro* was not high. This may have contributed to the high acclimatization rate of the *Kalmia* plants from PA-TIS (Fig. 5.9, p. 107). The accumulation of biomass as starch reserves during the *in vitro* rooting stage is important for plants' survival during hardening phase (Capellades et al., 1991). Escalona et al. (1999) reported that the survival rate *ex vitro* of pineapple shoots grown in TIS increased linearly with shoot size before the transfer. Since the *Kalmia* shoots did not grow well on WPM agar (+) (Fig. 5.33, p. 105), the accumulated biomass might not have been high enough, even when roots were formed, which corresponds to the results of Escalona et al. (1999).

6.4.4 Influence of relative humidity on rooting and acclimatization success

The generally high relative humidity *in vitro* has been made responsible for the malfunction of stomata after the *ex vitro* transfer (see Chapter 6.3.4). The higher survival rate (89 %) 15 days after the transfer of photoautotrophically cultivated *Coffea arabusta* was attributed to the lower relative humidity (85 - 90 %) in the TRI-bioreactor than in either RITA® (survival: 33 %, rH = 95-99 %) or the photomixotrophic control vessel (survival: 67 %, rH = 95 %). Similar results were presented by Zobayed et al. (2000b), where the acclimatization success after the PA-treatment in a self-built reactor was higher (survival: 86 %, rH = 85 - 91 %) than in the PM control (survival: 46 %, rH = 95 - 99 %). In the present study, fully functioning stomata were detected for 'Holsteiner Cox' in the photoautotrophic treatments, whereas no closure was determined in the photomixotrophic control (Fig. 5.26, p. 96). The stomata of *Kalmia* were not analyzed, but since the survival rate was indeed higher for the plants deriving from the photoautotrophic treatments (89 %), it may be assumed that the stomata functionality played a part in preventing high water losses *ex vitro*.

Ziv (1995) stated that the environment for plants propagated *in vitro* should be designed to support the normal development of leaves or roots. Only if the plants are photosynthetically active and able to regulate transpiration, they will survive the transfer to greenhouse conditions. In the present study, the approach of lowering the relative humidity in PA-TIS by bottom cooling was successful, and additionally the *Kalmia* shoots were able to accumulate enough carbohydrate through photosynthesis to acclimatize at a higher rate than the conventionally cultivated plants on WPM agar (+).

7 Conclusion and Outlook

The combination of a photoautotrophic cultivation with a temporary immersion system was successfully established in PA-TIS for the rooting of woody plants. The PA-TIS showed promising results in the rooting of both *Malus* and *Kalmia* shoots. The rooting percentage for the 'Holsteiner Cox' plantlets was not 100 %, but by varying some of the *in vitro* growth factors - lighting, medium composition, temperature - valuable insights were gained on the possibilities of influencing both plant quality and rooting efficiency *in vitro*. These could be used to optimize the protocol for rooting *Kalmia*, which had proven to be difficult to root in previous studies. The rooting efficiency could be improved from 23 % on agar containing sugar to 89 % in PA-TIS. Additionally, a higher acclimatization rate was realized for the photoautotrophically cultivated *Kalmia*, which can be attributed to the higher rooting efficiency, higher fresh weight accumulation, and induction of stomatal closure in PA-TIS.

The application of a system such as PA-TIS in commercial plant tissue culture, even if it has shown to be more successful than the conventional treatment, is not yet imaginable. In the present study, only 3 vessels, including 55 plants each, had to be concurrently monitored and regulated, whereas more units would have to be controlled in large laboratories. The continuous monitoring of carbon dioxide and relative humidity in the cultivation vessels was only possible with an IR-CO_2/H_2O-analyzer, of which a higher number would have to be acquired for a large-scale cultivation. In order to realize such a scale-up of PA-TIS, the following criteria have shown to be of interest:

Cultivation vessel

In the present study, directly ventilated vessels were used, which were small enough to still be autoclavable. The vessels consisted of polycarbonate and could be used multiple times. The cleaning of the vessels was not complicated, since they were heat-resistant and could be rinsed in a dishwasher. To date, no tarnishing of the material was detected. The design of PA-TIS as a box with a detachable lid was user-friendly, because vessel with screwtops (e.g. RITA®) are sometimes hard to open under sterile conditions. They are also not gas-proof, resulting in high gas losses.

It is imaginable that even larger vessels with a similar design to the 'Bio-Safe Carrier' could be used for PA-TIS, but then sterilization would have to be realized with chemicals and the plant transfer could not take place under a clean-bench. The plant carrier designed for PA-TIS (Fig. 4.2, p. 43) was ideal for larger plants when the leaves could be fixed,

but small *Kalmia* shoots were not kept in place. A carrier with a higher number of more flexible knobs (e.g. silicone) could for instance be integrated into PA-TIS for fixation.

Ventilation

The induction of photoautotrophy improved rooting and acclimatization of *Kalmia* significantly. Therefore, ventilation should always be applied to ensure carbon dioxide enrichment and to decrease of the relative humidity *in vitro*. The continuous measurement of the carbon dioxide concentration in a closed-loop, as introduced in this study, will not be possible if a high number of vessels has to be supplied. The enrichment of the cultivation room has already been suggested in different studies, where either filters were added to increase the gas exchange or the vessel was directly ventilated with enriched air. The pre-mixture of air and carbon dioxide in a smaller gas tank to save resources is also conceivable. Thereby, a closed-loop aeration of several vessels could still be maintained, but only the gas tank would be needed to be monitored. Here, a measurement for ethylene could be integrated and, if necessary, a gas-purge could take place. The net photosynthetic rate could still be determined per vessel, if a separate gas redirection to an IR-analyzer were implemented for each vessel.

Lighting

In the present study, not only the light intensity, but also the duration and direction of lighting was varied. The lateral lighting in the cultivation of 'Holsteiner Cox' in PA-TIS led to irregular growth and rooting, because it was only applied from two sides (Fig. 5.9, p. 76). The high light intensities, however, were beneficial for the photoautotrophic cultivation of both 'Holsteiner Cox' and *Kalmia*, with higher rooting efficiencies and fresh weight accumulation. In the future, a more flexible lighting from all sides with the help of LEDs is imaginable. The heat emission of LEDs is lower than of the fluorescent lamps used in the present study (Heo et al., 2002b; Sivakumar et al., 2006), so that the cultivation air would not be influenced so strongly. A flexible lateral lamp rack, mounted on hinges, could be equipped with LEDs, enabling easy access to the cultivation vessels when needed. The continuous lighting of 'Holsteiner Cox' resulted in high rooting, but to problems with photosynthesis. A change to a short light/dark-cycle of 4/2 h, as suggested by Morini and Perrone (2006) for *Malus*, could be tested in PA-TIS.

Medium

The immersion cycles were not varied in the present study. Other studies (e.g. by Damiano et al. (2002)) suggest that the dry matter content is significantly influenced by the

duration of medium contact. The occurrence of hyperhydricity, which was sometimes detected in PA-TIS, could thereby be reduced.

The removal of vitamins from the medium composition proved to influence the growth and rooting negatively. A culture completely free of carbon sources would also mean the total omission of plant growth regulators, which was not carried out in this study. Since no contamination occurred in PA-TIS when vitamins were present, nothing is to be said against using both growth regulators and vitamins to promote plant and root growth. Since the medium bottle can simply be exchanged, a combination of multiplication and rooting stage in a single PA-TIS treatment is imaginable, making the transfer in between the steps redundant

The *Kalmia* shoots on sugar-free agar medium exhibited a lower contamination rate than on WPM agar (+). Nonetheless, bacteria were found close to the plantlets, independent whether sugar was present or not (Fig. 5.34, p. 105). One of the arguments for photoautotrophy is the reduction of contamination. It would hence be interesting to determine what kind of bacteria were present in the *Kalmia* culture and which plant excretion provided breeding ground for them. It would also be interesting to test whether the removal of vitamins and plant growth regulators would have a visible effect on the bacterial growth as compared to the omission of solely sugar.

Temperature and relative humidity

Under high temperatures, a lower net photosynthetic rate was detected in PA-TIS. Consequently, higher fresh and dry weight accumulation was measured under lower temperatures. The use of bottom cooling in PA-TIS did not only lower the temperature, but also the relative humidity. The application of bottom cooling for other TIS applications is strongly recommended, because hyperhydricity can thereby be reduced. The use of higher temperatures during the night (positive DIF) may additionally improve plant quality and rooting success (Kozai et al., 1992a; Fujiwara and Kozai, 1995; Cui et al., 2000).

8 Summary

In the commercial plant production the possibilities of controlling cultivation vessels are limited. Normally, glass jars or clear plastic cups are used for the multiplication, elongation, and rooting of plant tissues. The environment, in which the plants grow, has to be influenced from the outside, so that room temperature, lighting, and ventilation have to be adapted to the plants' needs. In order to grow, the plants need a carbon source, which is mostly provided *in vitro* by adding sugar to the growth medium.

This harbors problems, since the addition of sugar allows for infections through bacteria and fungi that cannot always be prevented. The sealing of cultivation vessels against microorganisms results in limited air exchange, so that carbon dioxide cannot be replenished adequately. Also, the relative humidity within the vessels rises due to evaporation of the medium and transpiration of the plants. This leads to physiological problems such as stomata malfunction and hyperhydricity. The losses, either through contamination *in vitro* or later poor acclimatization rates *ex vitro*, add to the production costs, which are already high through the labor-intensive steps of plant tissue production. The problems of contamination and poor acclimatization can be overcome by omitting sugars from the medium composition and lowering the relative humidity *in vitro*. The photoautotrophic potential of chlorophyllous explants has to be exploited therefore. To promote photosynthesis the carbon dioxide concentration has to be elevated and sufficient lighting has to be applied.

In the present study, a system was designed that combines temporary immersion and photoautotrophic cultivation (PA-TIS) in one vessel. In order to induce photoautotrophic growth, vessels containing 55 plantlets each were equipped with a closed-loop ventilation, which could be enriched with carbon dioxide to a concentration above 1200 µmol mol^{-1}. Additionally, high light intensities (maximum PPFD 150 µmol m^{-2} s^{-1}) were applied either from above and two sides of the vessel or solely from above. The light cycle could be varied and was regulated to either 16/8 h light/dark or 24 h continuous lighting. The relative humidity in PA-TIS was lowered to under 90 %, reducing its negative influence on the plant tissue. The temporary immersion made a substrate-free cultivation possible, so that overprovision of the plant material with water was averted and transfer to soil simplified. A simultaneous control of three PA-TIS vessels was also implemented.

PA-TIS was successfully established for the rooting of the woody plants *Malus x domestica* Borkh. 'Holsteiner Cox' and *Kalmia latifolia* 'Ostbo Red'. The cultures were compared

to reference cultures in suspension and on agar, with or without the addition of sucrose. Although the rooting efficiency of 'Holsteiner Cox' in the photomixotrophic reference culture was higher (80 to 100 %) at first, the efficiency in PA-TIS could be improved to 96 % by increasing the light intensity to 150 µmol m^{-2} s^{-1} (continuous lighting).

The results for *Malus* helped to discern the influence of ventilation, lighting, temperature, relative humidity and medium composition on the plants' photosynthetic activity as well as on plant quality, growth, and rooting in photoautotrophic cultivation. The experiences with critical growth factors were transferred and implemented into the photoautotrophic rooting protocol of *Kalmia latifolia*, so that the rooting efficiency was in fact significantly higher in PA-TIS (89 %) than in the photomixotrophic reference culture (23 %) for *Kalmia*. Additionally, it was shown for the first time that *Kalmia* can be cultivated and rooted photoautotrophically. The acclimatization success upon the transfer *ex vitro* was also more successful for the photoautotrophic treatment (85 % instead of 62 %).

The application of direct regulation of growth factors *in vitro* has proven to be beneficial for the plant material. The carbon dioxide enrichment paired with high light intensities enabled photoautotrophic cultivation in PA-TIS. By continuously measuring the net photosynthetic rate through carbon dioxide uptake, an online monitoring of the plants' status (e.g. temperature-induced limitation of photosynthesis) was possible. Closed-loop ventilation, as applied here, may be an important tool for future investigations on photoautotrophy.

It was shown that stomatal functionality can be induced under photoautotrophic conditions, whereas malfunctions occurred in the photomixotrophic control. The lowering of the relative humidity by adding bottom cooling to the PA-TIS played an important part in that. No contamination arose in PA-TIS, whereas fungal and bacterial infections led to losses of *Malus* and *Kalmia* shoots (*in* or *ex vitro*). The application of temporary immersion proved to be especially beneficial for *Kalmia* since endophytes did not have the chance to grow without the continuous contact to plants and medium.

The combination of photoautotrophic and temporary immersion culture in the system introduced in this study may yet be too complicated to realize in commercial plant tissue production, but the good results for the difficult-to-root *Kalmia* may justify the investment in ventilation and temporary immersion equipment. For applied research, on the other hand, PA-TIS can be an important tool that provides many possibilities of influencing growth factors and measuring the immediate plant response (P_n or stomata).

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A Appendix

(a) (b)

Fig. A.1: Problems with the medium. (a) Discoloring of MS (-) medium in suspension culture; (b) fungal infection in MS agar (+) culture

(a) (b) (c)

Fig. A.2: Deformations of Holsteiner Cox shoots grown in PA-TIS: (a) underdeveloped shoot; (b) distorted stem; (c) thickened stem.

Fig. A.3: Plants lost in PA-TIS due to medium pump malfunction

A.1 Tables

A.1.1 Equipment

Device	**Model**	**Producer / Distributor**
Autoclave	V-100	Systec
CO_2/H_2O-Sensor	LI-COR 7000	LI-COR
Data acquisition	LabJack U12	Meilhaus
Digital camera	Coolpix P50	Nikon
Dry bead sterilizer	Steri 350	Sigma
Drying oven	T6	Heraeus
Flow meter		Aalborg
Fluorescent lamps	Lumilux 865. 18 W	Osram
Microscope	Primo Star LED	Zeiss
pH meter	pH 211	Hanna
Precision scales		Kern
Pumps		KNF
Relays	ME-UBRE	Meilhaus
Shaker	KS 15A	Edmund Bühler
Sterile work bench		Cleanair

A.1.2 Consumables

		Producer / Distributor
Bio-Safe Carrier		Nalgene
Cultivation vessels	Crystal clear, d = 10 cm	Hutahmaki
FEP hoses	d = 4 mm	Roth
Twist-off jars	150 ml	
Medium bottle	2.5 l	Schott
Polypropylene tubes	d =4 mm	HJ-Bioanalytik
PTFE hoses	d = 4 mm	Roth
Silicon hoses	d = 10 mm	Roth
Sterile filters	Midisart 2000, 0.2 μm	Sartorius

A.1.3 Chemicals and reagents

	Producer / Distributor
BAP (6-Benzylaminopurine)	Duchefa
Carbon dioxide	Air liquide
Ethanol (70 %)	Roth
Hydrochloric acid	Roth
IBA (Indole-3-butyric acid)	Duchefa
Magnesium perchlorate	Fisher Scientific
Murashige and Skoog salts	Duchefa
Murashige and Skoog salts incl. vitamins	Duchefa
Murashige and Skoog vitamins	Duchefa
Myo-inositol	Duchefa
Nitrogen	Air liquide
Potassium hydroxide	Roth
Plant Agar	Duchefa
Soda lime	VWR
Sucrose	Nordzucker
Super glue	Metaflux
Turf substrate TKS I	Floragard
WPM (Lloyd and McCown) salts	Duchefa
WPM (Lloyd and McCown) vitamins	Duchefa

A.1.4 Literature review

Tab. A.1: Chronological overview on cultivars micropropagated in temporary immersion systems

Publication	Cultivar	Tissue	Vessel type
Alvard et al. (1993)	*Musa acuminata*	meristem	Nalgene® filter
Cabasson et al. (1997)	*Citrus deliciosa*	somatic embryos	Nalgene® filter
Etienne et al. (1997)	*Hevea brasiliensis*	somatic embryos	Nalgene® filter
Lorenzo et al. (1998)	*Saccharum*	shoots	twin-flask
Escalona et al. (1999)	*Ananas comosus*	shoots	twin-flask
Jiménez et al. (1999)	*Solanum tuberosum*	microtubers	twin-flask
Akula et al. (2000)	*Camellia sinensis*	somatic embryos	Nalgene® filter
Young et al. (2000)	*Phalaenopsis*	protocorm-like bodies	BTBB
Martre et al. (2001)	*Hevea brasiliensis*	callus	RITA®
Barry-Etienne et al. (2002)	*Coffea arabica*	somatic embryos	RITA®
Espinosa et al. (2002)	*Ananas comosus*	callus	twin-flask
Gómez Kosky et al. (2002)	Banana hybrid	somatic embryos	twin-flask
Chakrabarty et al. (2003)	*Malus* M9 EMLA	nodal explants	BTBB
Pérez et al. (2003)	Pineapple	shoots	twin-flask
Rodríguez et al. (2003)	*Saccharum*	axillary shoots	twin-flask
Zobayed and Saxena (2003)	*Hypericum perforatum*	root sections	RITA®
Murch et al. (2004)	*Crescentia cujete*	plant nodes	RITA®
Zobayed et al. (2004b)	*Scutellaria baicalensis*	stem sections	RITA®
Albarrán et al. (2005)	*Coffea arabica*	somatic embryos	Nalgene® filter
Aragón et al. (2005)	*Musa* AAB	shoots	twin-flask
González-Olmedo et al. (2005)	*Ananas comosus* Merr.	shoots	twin-flask
Hanhineva et al. (2005)	*Fragaria* × *ananassa*	leaf mass	RITA®
Ilczuk et al. (2005)	*Hippeastrum* × *chmielii*	bulblets	twin-flask
Jova et al. (2005)	*Dioscorea* spp.	microtubers	Nalgene® filter
McAlister et al. (2005)	*Eucalyptus* clones	shoots	RITA®
Roels et al. (2005)	*Musa* AAB	shoots	twin-flask
Wawrosch et al. (2005)	*Charybdis*	nodules	twin-flask
Zhu et al. (2005)	*Malus* M26	shoots	RITA®
Dewir et al. (2006)	*Spathiphyllum cannifolium*	apical meristems	BTBB
Quiala et al. (2006)	*Cymbopogon citratus*	shoots	twin-flask
Roels et al. (2006)	*Musa* AAB		Nalgene® filter
Chakrabarty et al. (2007)	*Malus* M9 EMLA	single node cuttings	BTBB
Ducos et al. (2007)	*Coffea canephora*	somatic embryos	twin-flask
Niemenak et al. (2008)	*Theobroma cacao*	somatic embryos	twin-flask
Yang and Yeh (2008)	*Calathea orbifolia*	bud clusters	Plantima
Mordocco et al. (2009)	*Saccharum* spp.	transverse sections	RITA®

Tab. A.2: Chronological overview on cultivars micropropagated in photoautotrophic cultures

Publication	**Cultivar**
Kozai et al. (1987)	*Cymbidium*
Fujiwara et al. (1988)	Strawberry (*Fragaria* × *ananassa*)
Kozai et al. (1988)	*Solanum tuberosum*
Kozai et al. (1990)	*Cymbidium*
Kozai et al. (1991)	Strawberry (*Fragaria* × *ananassa* Duch.)
Kirdmanee et al. (1992)	*Cymbidium* protocorm-like bodies
Kirdmanee et al. (1995a)	*Eucalyptus camaldulensis*
Kirdmanee et al. (1995b)	*Eucalyptus camaldulensis*
Heo et al. (1996)	*Cymbidium*
Miyashita et al. (1996)	*Solanum tuberosum*
Tichá et al. (1998)	*Nicotiana tabacum*
Adelberg et al. (1999)	*Cucumis melo*
Afreen et al. (1999)	*Ipomoea batatas* L. (Lam), cv. Beniazuma
Cristea et al. (1999)	*Chrysanthemum*
Nguyen et al. (1999b)	*Coffea arabusta*
Zobayed et al. (1999a)	*Solanum tuberosum* L., cv. Benimaru
Zobayed et al. (1999c)	*Brassica oleracea*L.
Cui et al. (2000)	*Rehmannia glutinosa*
Nguyen et al. (2000)	*Discorea alata*
Seon et al. (2000)	*Rehmannia glutinosa*
Zobayed et al. (2000a)	*Ipomoea batatas*
Zobayed et al. (2000b)	*Eucalyptus camaldulensis*
Afreen et al. (2001)	*Coffea arabusta*
Hahn and Paek (2001)	Cymbidium, Neofinetia, Phalaenopsis
Heo et al. (2001)	*Ipomoea batatas* L. (Lam), cv. Beniazuma
Kubota et al. (2001)	*Lycopersicon esculentum* Mill.
Nguyen et al. (2001)	*Coffea arabusta*
Nguyen and Kozai (2001a)	Banana *Musa* spp.
Zobayed et al. (2001a)	*Brassica oleracea* and *Nicotiana tabacum*
Afreen et al. (2002a)	*Coffea arabusta*
Sha Valli Khan et al. (2002)	*Eucalyptus tereticornis* Smith
Lian et al. (2002)	Statice (*Limonium* 'Misty Blue')
Pruski et al. (2002)	*Solanum tuberosum* L. 'Russet Burbank'
Mei-Lan et al. (2003)	*Lilium* bulblets
Morini and Melai (2003)	*Malus pumila* hybrid *paradisiaca* × Northern Spy
Xiao et al. (2003)	*Saccharum* spp.
Xiao and Kozai (2004)	*Zantedeschia elliottiana* and *Cunninghamia lanceolata*
Tanaka et al. (2005)	*Eucalyptus (urophylla* × *grandis*)
Couceiro et al. (2006)	St. John's Wort (*Hypericum perforatum* L.)
Teixeira da Silva et al. (2006)	*Spatiphyllum* 'Merry'
Xiao and Kozai (2006)	*Limonium latifolium*
Valero-Aracama et al. (2007)	*Uniola paniculata* L.
Damiani and Schuch (2009)	*Vaccinium ashei* 'Delite'
Zhang et al. (2009)	*Mormodica grosvenori*

Tab. A.3: Overview on applied growth factors in photoautotrophic cultures: Photosynthetic photon flux density (PPFD), carbon dioxide concentration (CO_2), type of ventilation (active (A) or passive (P)), relative humidity (rH) and temperature (T)

Publication	**PPFD** (μmol m^{-2} s^{-1})	**CO_2** (μmol mol^{-1})	**Ventilation** (A/P)	**rH** (%)	**T** (°C)
Kozai et al. (1987)	230	975	P		25
Fujiwara et al. (1988)	96	315	A	93	25
Kozai et al. (1988)	210, 400	1250, 1750	P		25
Kozai et al. (1990)	102, 226	1100, 3000	P		15 - 35
Kozai et al. (1991)	200	2000	P	75	25
Kirdmanee et al. (1992)	70	385	P	60	25
Kirdmanee et al. (1995a)	80	1200	P	65	25
Kirdmanee et al. (1995b)	100	1200	A	85	26
Heo et al. (1996)	50, 100	500, 1000	P	80	25
Miyashita et al. (1996)	150	1000	P		25
Tichá et al. (1998)	200	20000	P		25
Adelberg et al. (1999)	50, 100, 150	500, 1000, 1500	P	70	21
Afreen et al. (1999)	150	950	P	77	26
Cristea et al. (1999)	250	20000	P		
Nguyen et al. (1999b)	150, 350	1450	P	75	28
Zobayed et al. (1999a)	150	1150	P	80	25
Zobayed et al. (1999c)	150	1050	A	92	28
Cui et al. (2000)	70, 140, 210	1000	P	70	25
Nguyen et al. (2000)	150	550	A	60	24
Seon et al. (2000)	140	2000	P	70	25
Zobayed et al. (2000a)	120	875	A	88	25
Zobayed et al. (2000b)	120	850	A	88	26
Afreen et al. (2001)	100	1000	P	82	25
Hahn and Paek (2001)	100	1000	P	70	25
Heo et al. (2001),	150	1500	A	80	29
Kubota et al. (2001)	100, 130	1300, 1500	P	80	24
Nguyen et al. (2001)	150, 250	1100	A	70	24
Nguyen and Kozai (2001a)	100, 200	1340	P	70	24
Afreen et al. (2002a)	100	1000	A	82	23
Sha Valli Khan et al. (2002)	125	1450	P	82	22
Lian et al. (2002)	50, 100, 200	1500	P	70	25
Pruski et al. (2002)	150	1500	P		20
Mei-Lan et al. (2003)	30, 60, 120	1500	P	70	25
Morini and Melai (2003)	210	$\leq$ 11000	A		24
Xiao et al. (2003)	100 - 400	1500	P	72	27
Xiao and Kozai (2004)	50 - 100	1500	A	88	22
Tanaka et al. (2005)	45	1000 - 3000	P		25
Couceiro et al. (2006)	150	1000	P	65	25
Teixeira da Silva et al. (2006)	30 - 90	3000	P		25
Xiao and Kozai (2006)	50 - 100	1500	P	80	25
Valero-Aracama et al. (2007)	200 - 400	1500	P	80	25
Damiani and Schuch (2009)	42		P		25
Zhang et al. (2009)	25, 50, 100, 200	1000	P	80	25

A.1.5 Results

A.1.5.1 Carbon dioxide

Tab. A.4: Results for the regression of P_n (sigmoidal trend, 3 parameter) for the treatments under different PPFD

	$f = \frac{a}{1+e^{-\frac{(x-x_0)}{b}}}$			
PPFD $(\frac{\mu mol}{m^2 s})$	a	b	x_0	R^2
115 + 75	4.0006	3.4115	4.2666	0.7058
115 + 100	2.7227	4.0947	3.1116	0.8223
150	5.3713	8.1502	11.4600	0.7722

Tab. A.5: Results of the linear regression for the decrease of carbon dioxide for one of three PA-TIS vessels: $C_{in}(t) = \Delta_{CO_2} t + C_{in}(t = 0)$. Division into day (from 06:00 to 22:00) and night (from 22:00 to 06:00)

	Δ_{CO_2} (μmol mol^{-1})		
Period	00:00 - 06:00 night	06:00 - 22:00 day	22:00 - 00:00 night
1	9.11		
2	-1.93		
3		-10.93	
4		-5.08	
5		-8.74	
6		-6.91	
7		-6.77	
8		-10.25	
9		-6.50	
10		-6.49	
11		-9.03	
12		-8.01	
13		-7.13	
14		-9.38	
15		-6.84	
16		-7.30	
17			3.45
AM		-7.81	
SD		1.64	

Tab. A.6: Results of the exponential regression for the decrease of carbon dioxide for a single day of the cultivation of 'Holsteiner Cox' : $C_{in}(t) - C_{out} = Ae^{-\alpha t}$. Division into day (from 06:00 to 22:00) and night (from 22:00 to 06:00).

Period	00:00 - 06:00		06:00 - 22:00		22:00 - 00:00	
	α	A	α	A	α	A
1	0.0018	1064				
2	0.0030	1655				
3			0.0152	1577		
4			0.0150	1566		
5			0.0142	1455		
6			0.0138	1430		
7			0.0138	1439		
8			0.0120	1411		
9			0.0136	1474		
10			0.0138	1456		
11			0.0144	1518		
12			0.0142	1482		
13			0.0156	1626		
14			0.0138	1498		
15			0.0140	1483		
16			0.0145	1548		
17			0.0135	1484		
18			0.0146	1584		
19			0.0145	1569		
20			0.0145	1544		
21			0.0151	1584		
22			0.0143	1540		
23			0.0150	1618		
24			0.0151	1604		
25			0.0155	1577		
26			0.0136	1545		
27					-0.0054	1463
28					0.0025	1631
AM			0.0143	1526		
SD			0.0008	63		

Tab. A.7: Results of the linear regression for the decrease of carbon dioxide for a single day of the cultivation of Holsteiner Cox: $C_{in}(t) = \Delta_{CO_2} t + C_{in}(t = 0)$. Division into day (from 06:00 to 22:00) and night (from 22:00 to 06:00).

Period	00:00 - 06:00	06:00 - 22:00	22:00 - 00:00
	Δ_{CO_2}	Δ_{CO_2}	Δ_{CO_2}
1	-1.6		
2	6.2		
3	-4.0		
4		-16.7	
5		-15.8	
6		-14.9	
7		-15.1	
8		-15.2	
9		-13.2	
10		-15.5	
11		-15.3	
12		-15.8	
13		-15.6	
14		-16.6	
15		-15.6	
16		-15.7	
17		-16.3	
18		-16.2	
19		-16.7	
20		-16.8	
21		-16.8	
22		-17.1	
23		-16.0	
24		-17.3	
25		-17.4	
26		-17.4	
27		-15.6	
28			8.6
29			-3.6
AM		-16.0	
SD		0.959	

A.1.5.2 Net photosynthetic rate

Tab. A.8: Mean net photosynthetic and respiration rate for all treatments

PPFD	115 + 75		115 + 100		150		150 cont.
Day	Pn	Respiration	Pn	Respiration	Pn	Respiration	Pn
1	0.3	-2.1	1.0	-1.9	2.1	-1.2	2.6
2	1.3	-2.3	0.9	-2.0	1.1	-1.0	1.9
3	1.6	-2.4	1.3	-2.0	1.7	-1.7	2.6
4	2.1	-1.9	1.8	-2.3	1.1	-1.9	2.2
5	2.8	-2.0	1.6	-2.2	1.8	-1.5	2.1
6	3.0	-1.8	1.7	-2.4	1.4	-1.7	2.5
7	3.1	-1.8	2.4	-1.8	2.5	-1.3	2.7
8	2.8	-2.1	2.1	-1.9	2.4	-0.9	2.2
9	2.3	-2.1	2.3	-2.3	2.4	-1.2	1.3
10	3.0	-2.4	2.0	-2.4	2.1	-1.2	1.0
11	3.7	-2.3	1.9	-2.5	2.1	-1.6	1.0
12	3.9	-1.9	2.5	-2.5	2.9	-0.9	1.1
13	4.2	-1.7	2.5	-2.6	2.5	-1.4	0.6
14	3.0	-1.9	2.4	-2.6	3.1	-1.4	0.2
15	3.4	-2.2	2.6	-2.6	3.2	-1.2	0.8
16	3.2	-2.0	2.7	-2.7	3.3	-1.1	0.9
17	3.6	-1.7	2.8	-2.9	3.0	-0.9	2.4
18	4.0	-1.8	2.6	-2.9	3.1	-1.4	3.4
19	4.7	-1.6	2.7	-2.9	3.1	-1.3	2.7
20	4.2	-1.8	3.5	-2.9	4.4	-1.4	2.3
21	3.6	-1.9	3.0	-2.9	5.1	-1.2	0.9
22	5.0	-2.1	2.7	-3.0	5.5	-0.8	1.7
23	4.5	-2.5	2.4	-2.9	5.0	-1.1	1.3
24	4.6	-2.5	2.6	-2.9	4.8	-1.6	1.6
25	4.8	-2.7	2.6	-2.9	5.4	-1.8	1.7
26	3.6	-2.6	2.4	-2.9	4.6	-1.4	1.6
27	2.4	-2.7	2.5	-3.0	4.0	-1.9	1.5
28	3.9	-2.6	2.8	0.0	3.2	-2.1	1.2

Tab. A.9: Net photosynthetic rate, respiration, and CO_2 assimilation for a Holsteiner Cox culture in one of the PA-TIS vessels

Day of cultivation	$\mathbf{P}_n$ (μmol h^{-1} $plant^{-1}$)	$\mathbf{R}_d$	$\mathbf{CO}_2$ (μmol)
0	2.5	-0.2	2023
1	0.8	0.0	685
2	0.6	-1.3	-58
3	0.1	-1.7	-660
4	0.2	-1.8	-601
5	0.5	-1.7	-364
6	3.4	0.0	3001
7	3.1	-0.2	2663
8	3.4	-1.5	2328
9	3.4	-0.9	2576
10	3.5	-1.4	2430
11	3.5	-0.4	2896
12	3.8	-0.7	3021
13	3.8	-1.7	2542
14	4.1	-1.0	3167
15	4.2	-0.4	3510
16	4.3	-0.9	3417
17	4.3	-1.5	3105
18	4.3	-1.6	3058
19	4.7	-0.6	3884
20	4.1	-1.0	3199
21	4.4	-1.0	3422
22	4.9	-1.2	3801
23	4.5	-1.5	3297
24	4.8	-1.6	3509
25	4.3	-1.7	3008
26	5.3	-1.2	4106
27	4.5	-1.7	3197
28	4.5	-1.9	3143
		Sum (μmol CO_2)	**73427**
		Sum (mg CO_2)	**3226**

A.1.5.3 Relative humidity

Tab. A.10: Mean relative humidity in all PA-TIS vessels during the cultivation of HC and KL

PPFD ($\frac{\mu mol}{m^2 s}$)	**Run**	**Day rH** (%)		**Night rH** (%)	
		HC	KL	HC	KL
115 + 75	1	81.7 ± 3.3		80.1 ± 3.2	
	2	75.0 ± 2.1		71.9 ± 3.8	
	3	53.6 ± 17.2		46.0 ± 15.5	
115 + 100	1	75.5 ± 3.2		72.7 ± 5.2	
	2	74.5 ± 2.8		66.8 ± 3.6	
	3	74.2 ± 2.7		71.8 ± 4.5	
150	1	79.2 ± 1.3	78.5 ± 0.9	78.1 ± 1.4	75.3 ± 2.0
	2	81.0 ± 2.0	82.1 ± 1.5	78.5 ± 3.5	79.7 ± 1.9
	3	77.5 ± 1.3	83.0 ± 1.1	73.5 ± 1.6	81.3 ± 1.5
150 (cont.)	1	81.8 ± 3.5			
	2	80.9 ± 3.6			
	3	67.3 ± 7.0			

A.1.5.4 Temperature

Tab. A.11: Mean temperature in all PA-TIS vessels during the cultivation of HC and KL

PPFD (μmol m^{-2} s^{-1})	**Run** (vessels)	**T**$_{PA-TIS}$ (day, °C)	**T**$_{PA-TIS}$ (night, °C)	**DIF** (K)	**T**$_{room}$ (day, °C)	**T**$_{room}$ (night, °C)	**DIF** (K)
'Holsteiner Cox'							
115 + 75	1 (1)	24.1 ± 0.6	24.1 ± 0.6	0	22.2 ± 0.7	21.2 ± 0.6	1.0
	2 (1)	24.8 ± 0.8	24.7 ± 0.8	0.1	23.8 ± 0.8	22.1 ± 0.8	1.7
	3 (1)	25.1 ± 0.5	24.9 ± 0.5	0.2	28.2 ± 1.2	22.2 ± 0.4	6.1
115 + 100	1 (1)	26.0 ± 1.1	26.0 ± 0.9	0.5	23.6 ± 0.8	23.3 ± 1.1	0.4
	2 (1)	26.1 ± 1.5	25.7 ± 1.7	0.5	22.9 ± 1.5	22.3 ± 1.7	0.7
	3 (1)	28.2 ± 2.0	27.6 ± 2.1	0.6	25.6 ± 2.0	24.4 ± 2.1	1.2
150	1 (2)	25.7 ± 1.1	25.1 ± 0.9	0.6	23.7 ± 1.1	22.4 ± 1.0	1.3
	2 (2)	24.1 ± 0.5	23.8 ± 0.7	0.4	22.3 ± 1.0	21.1 ± 1.1	1.2
	3 (3)	23.1 ± 1.0	22.7 ± 0.9	0.4	20.7 ± 0.4	19.8 ± 0.4	0.9
150 (cont.)	1 (3)	25.8 ± 1.2	-	-			
	2 (2)	26.5 ± 0.5	-	-			
	3 (3)	27.1 ± 0.3	-	-			
Kalmia latifolia							
150	1 (1)	25.5 ± 1.0	24.9 ± 0.9	0.6	23.3 ± 1.6	22.1 ± 1.2	1.2
	2 (1)	24.1 ± 0.4	23.6 ± 0.4	0.5	22.3 ± 0.6	21.2 ± 0.5	1.2
	3 (1)	23.5 ± 0.4	22.9 ± 0.4	0.5	21.5 ± 0.5	19.8 ± 0.3	1.7

A.1.5.5 Plant quality and rooting

Tab. A.12: Fresh weight, dry weight and dry weight content (DMC) of 'Holsteiner Cox' after 4 weeks in the reference cultures and PA-TIS. Arithmetic mean and standard deviation for every run were calculated per shoot.

Treatment	Run	N° of shoots	Vitamins	PPFD ($\frac{\mu mol}{m^2 s}$)	Fresh weight (mg)	Dry weight (mg)	DMC (%)
FIRST SETUP							
MS susp. (+)	all	120*	+	75	871 ± 80	90 ± 10	10.3 ± 1.3
MS susp. (-)	all	120*	+	75	177 ± 19	15 ± 2	8.6 ± 0.6
MS agar (+)	all	120*	+	75	680 ± 52	80 ± 9	11.8 ± 0.7
MS agar (-)	all	120*	+	75	265 ± 36	25 ± 4	10.3 ± 1.5
PA-TIS (-)	all	152*	+	115 + 75	480 ± 108	55 ± 12	11.5 ± 1.0
MS agar (+)	all	120	+	75	784 ± 215	88 ± 20	11.5 ± 2.0
MS agar (-)	all	120	+	75	270 ± 90	32 ± 11	12.0 ± 2.1
PA-TIS (-)	all	165	+	115 + 100	604 ± 268	67 ± 40	10.8 ± 2.0
SECOND SETUP							
MS agar (+)	1	40	-	75	465 ± 160	50 ± 16	11.2 ± 2.1
MS agar (+)	2	30	-	75	239 ± 75	40 ± 11	17.3 ± 2.7
MS agar (+)	3	40	-	75	249 ± 85	29 ± 9	16.3 ± 3.2
MS agar (-)	1	40	-	75	132 ± 69	15 ± 9	12.0 ± 4.3
MS agar (-)	2	30	-	75	107 ± 34	18 ± 7	16.1 ± 5.1
MS agar (-)	3	40	-	75	117 ± 38	19 ± 4	15.0 ± 3.4
PA-TIS (-)	1	109	-	150	352 ± 167	43 ± 23	12.0 ± 3.1
PA-TIS (-)	2	110	-	150	319 ± 109	53 ± 21	17.4 ± 4.8
PA-TIS (-)	3	165	-	150	476 ± 199	42 ± 22	11.0 ± 2.3
MS agar (+)	1	20	-	75 (cont.)	387 ± 171	62 ± 25	16.5 ± 2.2
MS agar (+)	2	25	-	75 (cont.)	669 ± 239	84 ± 28	12.8 ± 1.5
MS agar (+)	3	30	-	75 (cont.)	513 ± 172	71 ± 23	14.0 ± 2.3
MS agar (-)	1	25	-	75 (cont.)	218 ± 148	28 ± 22	12.9 ± 4.4
MS agar (-)	2	20	-	75 (cont.)	170 ± 79	24 ± 10	14.8 ± 2.5
MS agar (-)	3	40	-	75 (cont.)	128 ± 58	22 ± 11	17.0 ± 3.8
PA-TIS (-)	1	163	-	150 (cont.)	585 ± 268	76 ± 40	13.0 ± 3.1
PA-TIS (-)	2	110	-	150 (cont.)	637 ± 238	81 ± 47	12.2 ± 3.9
PA-TIS (-)	3	165	-	150 (cont.)	544 ± 236	77 ± 47	13.4 ± 4.1

* Fresh and dry weight were determined not for single shoots, but for all shoots of one repetition. These values were excluded from the statistical analysis in Fig. 5.7.

Tab. A.13: Root number and length per rooted 'Holsteiner Cox' shoot after 4 weeks in the reference cultures and PA-TIS. Arithmetic mean and standard deviation for every run were calculated per shoot.

Treatment	**Run**	**N° of shoots**	**Vitamins**	**PPFD** ($\frac{\mu mol}{m^2 s}$)	**Number of roots** (mg)	**Root length** (cm)
FIRST SETUP						
MS susp. (+)	all	120	+	75	-	-
MS susp. (-)	all	120	+	75	-	-
MS agar (+)	1	40	+	75	11.7 ± 3.2	3.0 ± 0.7
MS agar (+)	2	40	+	75	11.9 ± 3.4	3.0 ± 0.8
MS agar (+)	3	40	+	75	9.6 ± 3.2	3.8 ± 1.0
MS agar (-)	1	40	+	75	2.6 ± 2.5	1.7 ± 0.7
MS agar (-)	2	32	+	75	3.0 ± 1.4	1.3 ± 0.9
MS agar (-)	3	40	+	75	4.0 ± 2.6	0.5 ± 0.4
PA-TIS (-)	1	55	+	115 + 75	6.3 ± 2.6	1.8 ± 0.9
PA-TIS (-)	2	55	+	115 + 75	4.2 ± 2.3	3.3 ± 1.7
PA-TIS (-)	3	42	+	115 + 75	3.7 ± 2.0	0.6 ± 0.5
MS agar (+)	1	40	+	75	13.6 ± 3.1	5.2 ± 0.8
MS agar (+)	2	40	+	75	9.5 ± 3.1	4.9 ± 0.9
MS agar (+)	3	40	+	75	13.6 ± 3.6	3.1 ± 1.0
MS agar (-)	1	40	+	75	1.3 ± 0.5	0.5 ± 0.5
MS agar (-)	2	40	+	75	4.2 ± 2.4	0.4 ± 0.4
MS agar (-)	3	40	+	75	2.5 ± 2.0	0.4 ± 0.1
PA-TIS (-)	1	55	+	115 + 100	5.5 ± 3.6	2.0 ± 1.5
PA-TIS (-)	2	55	+	115 + 100	6.2 ± 2.7	2.6 ± 1.2
PA-TIS (-)	3	55	+	115 + 100	4.2 ± 2.2	2.0 ± 1.2
SECOND SETUP						
MS agar (+)	1	40	-	75	9.5 ± 3.1	2.3 ± 0.9
MS agar (+)	2	30	-	75	5.6 ± 2.5	3.0 ± 0.9
MS agar (+)	3	40	-	75	6.1 ± 3.0	3.4 ± 0.9
MS agar (-)	1	40	-	75	3.1 ± 2.3	0.9 ± 0.6
MS agar (-)	2	30	-	75	1.5 ± 0.7	2.0 ± 0.6
MS agar (-)	3	40	-	75	1.0 ± 0.0	0.6 ± 0.5
PA-TIS (-)	1	109	-	150	5.6 ± 3.0	1.1 ± 0.7
PA-TIS (-)	2	110	-	150	5.5 ± 4.3	1.8 ± 1.0
PA-TIS (-)	3	165	-	150	5.7 ± 3.3	2.0 ± 1.1
MS agar (+)	1	20	-	75 (cont.)	4.6 ± 2.8	1.4 ± 0.9
MS agar (+)	2	25	-	75 (cont.)	10.4 ± 3.7	2.8 ± 0.6
MS agar (+)	3	30	-	75 (cont.)	9.6 ± 2.6	3.0 ± 0.7
MS agar (-)	1	25	-	75 (cont.)	3.1 ± 2.0	1.4 ± 1.1
MS agar (-)	2	20	-	75 (cont.)	2.4 ± 1.4	1.1 ± 0.6
MS agar (-)	3	40	-	75 (cont.)	2.7 ± 1.7	0.8 ± 0.5
PA-TIS (-)	1	163	-	150 (cont.)	8.9 ± 3.1	2.8 ± 1.1
PA-TIS (-)	2	110	-	150 (cont.)	7.2 ± 2.9	2.0 ± 1.3
PA-TIS (-)	3	165	-	150 (cont.)	6.9 ± 3.2	2.7 ± 1.1

A.1.6 Statistical analysis

Fresh weight per shoot

PA-TIS (115 + 100 μmol m^{-2} s^{-1})

The differences in the median values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.854).

PA-TIS (150 μmol m^{-2} s^{-1})

Comparison	Diff of Ranks	Q	P<0.05
PA-TIS 150-3 vs PA-TIS 150-2	85.269	6.208	Yes
PA-TIS 150-3 vs PA-TIS 150-1	64.708	5.085	Yes
PA-TIS 150-1 vs PA-TIS 150-2	20.561	1.368	No

PA-TIS (continuous 150 μmol m^{-2} s^{-1})

Comparison	Diff of Ranks	Q	P<0.05
PA-TIS 150c-2 vs PA-TIS 150c-3	45.714	2.934	Yes
PA-TIS 150c-2 vs PA-TIS 150c-1	35.255	2.257	No
PA-TIS 150c-1 vs PA-TIS 150c-3	10.459	0.748	No

Dry weight per shoot

PA-TIS (115 + 100 μmol m^{-2} s^{-1})

The differences in the median values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.163).

PA-TIS (150 μmol m^{-2} s^{-1})

Comparison	Diff of Ranks	Q	P<0.05
PA-TIS 150-2 vs PA-TIS 150-3	40.501	3.353	Yes
PA-TIS 150-2 vs PA-TIS 150-1	37.973	3.496	Yes
PA-TIS 150-1 vs PA-TIS 150-3	2.528	0.219	No

PA-TIS (continuous 150 μmol m^{-2} s^{-1})

The differences in the median values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.884).

Number of leaves per shoot

PA-TIS (115 + 100 μmol m^{-2} s^{-1})

The differences in the median values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.064).

PA-TIS (150 μmol m^{-2} s^{-1})

Comparison	Diff of Ranks	Q	P<0.05
PA-TIS 150-3 vs PA-TIS 150-2	78.305	5.638	Yes
PA-TIS 150-2 vs PA-TIS 150-1	61.977	4.122	Yes
PA-TIS 150-3 vs PA-TIS 150-1	16.329	1.282	No

PA-TIS (continuous 150 μmol m^{-2} s^{-1})

Comparison	Diff of Ranks	Q	P<0.05
PA-TIS 150c-1 vs PA-TIS 150c-3	122.663	8.857	Yes
PA-TIS 150c-1 vs PA-TIS 150c-2	107.114	6.953	Yes
PA-TIS 150c-3 vs PA-TIS 150c-2	15.548	1.031	No

Max. leaf length per shoot

PA-TIS (115 + 100 μmol m^{-2} s^{-1})

The differences in the median values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.087).

PA-TIS (150 μmol m^{-2} s^{-1})

Comparison	Diff of Ranks	Q	P<0.05
PA-TIS 150-2 vs PA-TIS 150-1	84.468	5.631	Yes
PA-TIS 150-3 vs PA-TIS 150-2	42.030	3.076	Yes
PA-TIS 150-3 vs PA-TIS 150-1	42.437	3.098	Yes

PA-TIS (continuous 150 μmol m^{-2} s^{-1})

The differences in the median values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.846).

Number of roots per shoot

PA-TIS (115 + 75 μmol m^{-2} s^{-1})			
PA-TIS (115 + 75)-1 vs PA-TIS (115 + 75)-3	25.030	3.570	Yes
PA-TIS (115 + 75)-1 vs PA-TIS (115 + 75)-2	21.256	3.300	Yes
PA-TIS (115 + 75)-2 vs PA-TIS (115 + 75)-3	3.774	0.505	No
PA-TIS (115 + 100 μmol m^{-2} s^{-1})			
Comparison	Diff of Ranks	Q	P<0.05
PA-TIS (115 + 100)-2 vs PA-TIS (115 + 100)-3	22.103	2.697	Yes
PA-TIS (115 + 100)-2 vs PA-TIS (115 + 100)-1	10.339	1.531	No
PA-TIS (115 + 100)-1 vs PA-TIS (115 + 100)-3	11.764	1.388	No
PA-TIS (150 μmol m^{-2} s^{-1})			
The differences in the median values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.323).			
PA-TIS (continuous 150 μmol m^{-2} s^{-1})			
Comparison	Diff of Ranks	Q	P<0.05
PA-TIS 150c-1 vs PA-TIS 150c-3	66.006	4.942	Yes
PA-TIS 150c-1 vs PA-TIS 150c-2	50.588	3.465	Yes
PA-TIS 150c-3 vs PA-TIS 150c-2	15.418	1.029	No

Max. root length per shoot

PA-TIS (115 + 75 μmol m^{-2} s^{-1})			
Comparison	Diff of Ranks	Q	P<0.05
PA-TIS (115 + 75)-2 vs PA-TIS (115 + 75)-3	49.558	6.647	Yes
PA-TIS (115 + 75)-2 vs PA-TIS (115 + 75)-1	21.787	3.345	Yes
PA-TIS (115 + 75)-1 vs PA-TIS (115 + 75)-3	27.771	3.974	Yes
PA-TIS (115 + 100 μmol m^{-2} s^{-1})			
Comparison	Diff of Ranks	Q	P<0.05
PA-TIS (115 + 100)-2 vs PA-TIS (115 + 100)-1	18.881	2.796	Yes
PA-TIS (115 + 100)-2 vs PA-TIS (115 + 100)-3	14.168	1.729	No
PA-TIS (115 + 100)-3 vs PA-TIS (115 + 100)-1	4.712	0.556	No
PA-TIS (150 μmol m^{-2} s^{-1})			
Comparison	Diff of Ranks	Q	P<0.05
PA-TIS 150-3 vs PA-TIS 150-1	80.065	6.665	Yes
PA-TIS 150-3 vs PA-TIS 150-2	15.263	1.292	No
PA-TIS 150-2 vs PA-TIS 150-1	64.803	5.062	Yes
PA-TIS (continuous 150 μmol m^{-2} s^{-1})			
Comparison	Diff of Ranks	Q	P<0.05
PA-TIS 150c-1 vs PA-TIS 150c-2	87.008	5.959	Yes
PA-TIS 150c-1 vs PA-TIS 150c-3	4.303	0.322	No
PA-TIS 150c-3 vs PA-TIS 150c-2	82.705	5.518	Yes

Curriculum Vitae

Personal data

Name	Stefanie Fuljahn
Address	Sallstr. 37
	30171 Hannover
Date of birth	09.04.1980

Schooling

Jul 1999	**Degree:** Abitur (1,2)
1992 - 1999	Gymnasium Wilhelm-Raabe-Schule, Hannover
	Bilingual classes till 1997

Academic background

Oct 2005 - to date	PhD studies in 'Horticultural Sciences'
	at the "Leibniz Universität Hannover"
Mar 2005	**Degree:** Diploma in 'Biotechnology' (1,4)
Mar 2005	Thesis: "Untersuchungen zum Einfluss von Kohlenstoff-
	dioxid und Sauerstoffarmut auf die Vitalität von
	A. thaliana" (AG Hehl, Genetics, TU Braunschweig)
Nov 2003	Seminar paper: "Untersuchung potentieller WRKY-6
	Bindungsstellen mittels transienter und stabiler
	Transformation in Arabidopsis thaliana"'
	(AG Hehl, Genetics, TU Braunschweig)
Oct 1999	Start of biotechnology studies
	at the "Technische Universtiät Braunschweig"

Publications

2010	S. Fuljahn, T. Winkelmann, H.-J. Tantau.(2010) Bewurzelung von *Kalmia latifolia* in einem photoautotrophen temporären Immersionssystem (PA-TIS). BHGL-Schriftenreihe Band 27, S. 132
2009	S. Fuljahn, H.-J. Tantau. (2009) Process Engineering as a means of regulating the microclimate in a photoautotrophic *in vitro* culture. Acta Horticulturae (ISHS) 817:143-150
2009	S. Fuljahn, H.-J. Tantau. (2009): Bewurzelung von Apfelsprossen in einem photoautotrophen temporären Immersionssystem (PA-TIS). BHGL-Schriftenreihe Band 26, S. 125
2005	R. Hehl, J. Grohmann, S. Fuljahn, R.R. Mendel, R. Cerff, R. Hänsch. (2005) Genetic approaches to investigate the high CO_2 response of plants during soil flooding. Recent Research Developments in Soil Science, 1:37-49

Practical experience

Jun 2005 - to date	Employment as research and teaching assistant at the Biosystems and Horticultural Engineering Section, Institute of Biological Production Systems, Leibniz Universität Hannover
2005	Private tutor for the 'Studienhilfe' in Lehrte
2003-2005	Student assistant at the TU Braunschweig, supervision of 6 practical courses in 'Molecular Genetics' for biotechnology and biology students
2001-2002	Student lab and computer assistant at the GBF (now HZI) (AG Weiß, Molecular Immunology)

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"No man is an island, entire of itself..."
(J. Donne)

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