Vitis 35 (4), 163–167 (1996)

Stomatal behaviour, photosynthesis and photorespiration of *in vitro*-grown grapevines: Effects of light and CO_2

by

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S u m m a r y : To improve photosynthesis and growth of grapevines cultivated *in vitro* (Seyval blanc and SO 4) effects of light intensity, spectral irradiance and CO_2 concentration on stomatal behaviour, CO_2 fixation and photorespiration were studied. Stomata were shown to respond to changes of light intensity but, unlike photosynthesis, their reactions were delayed and stomatal closure was incomplete in the dark. In contrast, alterations of the CO_2 concentration in the headspace (50-2200 ppm) did not cause stomatal reactions. Photosynthesis *vs.* light intensity relationships indicated lower light compensation points, higher quantum yield and higher rates of light-saturated photosynthesis with "Fluora" lamps (maximal spectral irradiance at 460 and 680 nm) compared to "projector" lamps (maximal spectral irradiance at 620 nm). Photosynthesis *vs.* intercellular CO_2 concentration relationships indicated varietal differences, the carboxylation efficiency and rates of photosynthesis at CO_2 saturation being distinctly higher in the more vigorous variety SO 4 compared to Seyval blanc. Under the usual light conditions of our *in vitro* culture (50-60 µmol quanta·m^{-2}·s^{-1}, Fluora) the headspace CO_2 concentration ranged from 145 to 155 ppm while at the end of a 10-hour dark period it increased to values >3000 ppm. Rates of photorespiration were high (>50 % of photosynthesis) due to the relative low CO_2 concentrations and, presumably, due to elevated O_2 concentrations in the headspace. It is concluded that the often observed low rates of photosynthesis of *in vitro* plantlets are mainly due to low light intensity and CO_2 concentration in the headspace, the latter depending on the low rates of gas diffusion between ambient air and headspace.

K e y w o r d s : in vitro culture, stomatal conductance, photosynthesis, dark respiration, photorespiration, light, CO_2.

Introduction

CO_2 and light conditions are of predominant importance for growth and development of plantlets in culture vessels as many *in vitro*-grown species, including grapevines, were shown to be photoautotrophic, i.e. to grow with CO_2 and light as the sole carbon and energy sources (review: Widholm 1995, grapevine: Lima da Silva *et al.* 1996). The CO_2 concentration in the aerial part of vessels containing plantlets, the headspace CO_2 concentration (ch), depends on the rate of CO_2 uptake and respiration of the plantlet, the ambient CO_2 concentration and the rate of CO_2 diffusion between ambient atmosphere and headspace. Rates of photosynthetic CO_2 uptake of *in vitro* plants have been shown to be similar to those of outdoor-grown plants when put on a chlorophyll basis but lower when put on a dry weight basis (Widholm 1995, Lima da Silva *et al.* 1996). Dark respiration rates of *in vitro* plants were shown to be higher compared to outdoor-grown plants (Chagvardieff *et al.* 1990). In tissue culture light intensity is often equal or lower than one tenth of the light intensity at which photosynthesis of outdoor-grown plants is light-saturated (ca. 800 µmol quanta·m^{-2}·s^{-1}, Düring 1988). Thus, although presumably adapted to low light intensity, higher light intensities are assumed to raise rates of photosynthesis and plant growth of *in vitro* plantlets (Kozai *et al.* 1990).

The number of experiments to improve photosynthesis and growth of *in vitro* grapevines by alterations of ambient conditions in the vessels, namely light intensity and CO_2 concentration, is rather limited. Fournioux and Bessis (1986) investigated effects of headspace CO_2 on the morphogenesis of grapevines. To our knowledge, Falque *et al.* (1991) and recently Lima da Silva *et al.* (1995, 1996) were the first to present relationships between rates of photosynthesis and CO_2 supply using *in vitro*-grown grapevines.

In this paper rates of photosynthetic CO_2 fixation as affected by light intensity and CO_2 concentration and rates of photorespiration of *in vitro*-cultivated grapevines were studied. There is evidence for stomata of *in vitro*-grown grapevines to respond to alterations of light intensity but not to changes of CO_2 concentration.

Materials and methods

P l a n t m a t e r i a l : *In vitro* plantlets of the interspecific hybrid Seyval blanc (Seibel 4995 x Seibel 4986) and the rootstock variety SO 4 (*Vitis berlandieri* x *Vitis riparia*) have been used in our studies. After transfer to *in vitro* conditions the cultures were kept under standardized ambient conditions of 26 ± 1 °C, 16 h light at an irradiance of ca. 50-60 µmol quanta · m^{-2} · s^{-1} provided by Fluora fluorescent lamps (Osram L58W/77). The cultures were maintained by routine subcultures in 3-month intervals onto fresh LS medium (Linsmaier and Skoog 1965) according to Harst (1995).

M e t h o d s : Plantlets were selected for uniformity and transferred to a room with similar ambient conditions, temperature ranging from 26 to 20 °C (day/night).

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For measurements of gas exchange light was provided by a projector lamp (12 V, 75 W, General Electric). Light intensity in the vessels was altered by inserting stepwise gray filters below the light bulb. Maximal spectral irradiance of this type of lamp is at 620 nm (orange).

Alternatively, light was provided by 8 Fluora lamps (Osram L 18W/77) grouped in front (3), behind (3) and on top of the vessel (2) containing the plantlet. Except where indicated we used Fluora lamps in our experiments (mean light intensity in the vessels: 162 ± 13 µmol quanta·m^{-2}·s^{-1}). Light intensity was altered by switching on or off one or more lamps alternately on the left and right hand side. Maximal spectral irradiance of Fluora lamps is at 460 nm (blue) and 680 nm (red). Darkness was obtained by switching off all lamps and by covering the vessel by a black plastic foil.

Gas exchange measurements were performed using a "Miniküvetten System" (Walz, Effeltrich, Germany) with a gas mixing device to keep the CO_2 concentration constant and to lower the O_2 concentration of the gas stream by using of N_2 instead of ambient air (details: DÜRING 1988). The glass lid of the culture vessels was replaced by a rubber seal containing two glass tubes through which the measuring gas entered and left the vessel. Temperature and humidity of the gas stream were kept at 22.0 ± 0.1 °C and 98-99 %, respectively. To keep the temperature of the gas stream constant the air passed through the temperature-controlled measuring cuvette and from here directly into the vessel. Air humidity was controlled by adjusting the dew point of the gas stream by Peltier elements (MGK-4, Walz). In a series of experiments the surface of the medium was covered by paraffin oil to prevent gas exchange between the root zone and the headspace. Data were collected by a data logger (DES, Walz) and calculated using DIAGAS software, version 1.38 (Walz).

To determine the CO_2 concentration of the headspace (c_h) vessels closed by a glass lid and containing a plantlet (Seyval blanc, shoot length 12.5 ± 0.4 cm, 0.07 ± 0.01 g dry weight) were perforated by a glass borer just above the medium (diameter of the hole: ca. 1.5 mm) and sealed by an elastic, air-tight foil (Parafilm). From 9 to 11 a.m., i.e. at least 2 h after the onset of the light period, when c_h values had equilibrated, air samples from the headspace were collected by a 50 ml syringe after perforating the elastic seal by the needle. The air samples were injected into a CO_2 analyser (Binos 4b.1, Leybold-Heraeus, Germany) measuring CO_2 concentrations up to 3000 ppm.

Rates of photorespiration of plant tissue were calculated according to SHARKEY (1988).

Results

Transpiration and stomatal reaction: It has been documented that stomata close if ambient CO_2 concentration is raised above a threshold, which depends on the CO_2 sensitivity of the leaf (DÜRING 1991, DÜRING and STOLL 1996). In our experiments with *in vitro* plantlets (Seyval blanc) rates of transpiration, which are closely related to stomatal aperture (RASCHKE 1979, DÜRING 1987) were not affected by ambient CO_2 increasing from 50 to 1200 ppm. In additional experiments, they even did not respond to headspace CO_2 concentrations increasing stepwise to 2200 ppm (data not shown).

In a subsequent experiment the light intensity provided by Fluora lamps was reduced stepwise from 190 µmol quanta·m^{-2}·s^{-1} to zero and increased again to ca. 190 µmol quanta·m^{-2}·s^{-1}. As is shown in Fig. 1 rates of photosynthesis reacted almost synchronously to changes of light intensity (r^2 = 0.77) and also transpiration decreased to 50 % in the dark; when light intensity was raised transpiration increased again. In contrast to photosynthesis responses of transpiration were much less synchronized to changes of light intensity (r^2 = 0.43).

These experiments indicate that stomata of *in vitro* plantlets are functional to a certain extent but will not close completely in the dark.

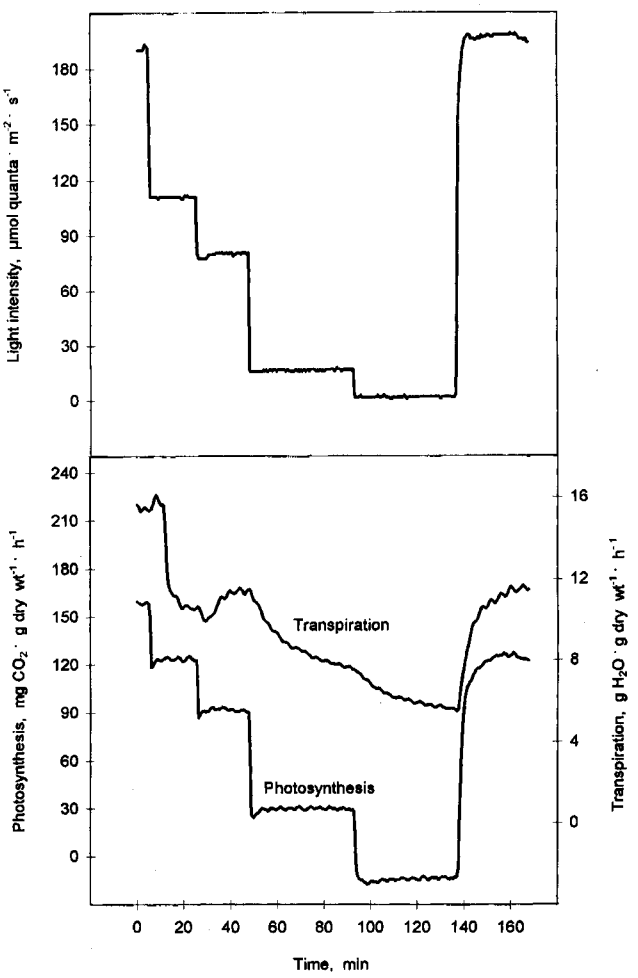

Fig. 1: Effects of stepwise alteration of light intensity provided by Fluora lamps (above) on rates of photosynthesis and transpiration indicating stomatal reactions. Variety: Seyval blanc.

Photosynthesis: To study the effects of light intensity on photosynthesis Seyval blanc and SO 4 plantlets were exposed to increasing light intensity (Fig. 2). With projector lamps the light compensation point ranged between 6 (SO 4) and 13 µmol quanta·m^{-2}·s^{-1} (Seyval blanc), thus being similar to outdoor-grown grapes (DÜRING 1988). With Fluora lamps the light compensation point was dis-

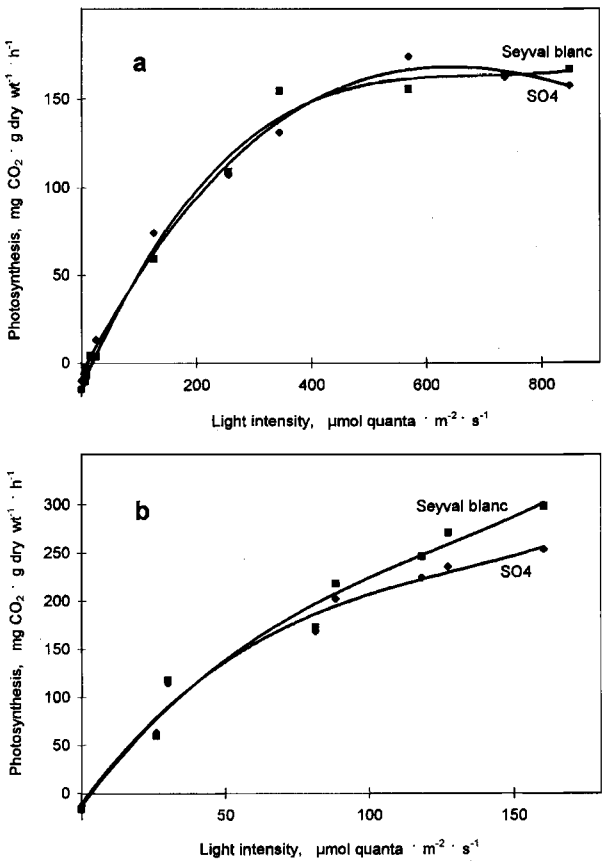

Fig. 2: Effects of light intensity provided by two different light sources, a projector lamp (**a**) and Fluora lamps (**b**), on photosynthesis of two varieties. Note, that graduations are different in **a** and **b**. Symbols represent average values of 4 replicates; varietal differences are not significant (P = 5 %).

tinctly lower, 2.6 µmol quanta·m^{-2}·s^{-1} for both varieties. Quantum yields (mg CO_2 · g dry wt^{-1}·h^{-1} related to µmol quanta·m^{-2}·s^{-1}) were equal in both varieties but differed with the type of lamp: 0.5 with the projector lamp and 3.0 with Fluora lamps. With the projector lamp light saturation was reached at values >600 µmol quanta·m^{-2}·s^{-1} in both varieties while with Fluora lamps it was not fully achieved due to the limited number of lamps installed. Obviously,

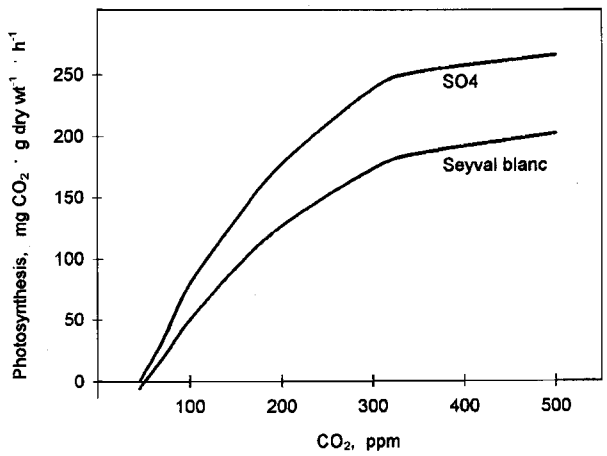

Fig. 3: The photosynthesis *vs.* headspace CO_2 concentration relationship of Seyval blanc and SO 4 vines. LSD (5 %) = 16.3.

light provided by Fluora lamps is more efficient with regard to photosynthesis than light provided by projector lamps.

Reactions of photosynthesis of *in vitro* plants to various CO_2 concentrations in the headspace were determined by increasing stepwise the CO_2 concentration from 50 to 2000 ppm at saturating light intensity, constant temperature and constant air humidity. In preexperiments with and without the medium being covered with paraffin oil we found that the CO_2 concentration of the headspace was not significantly altered by CO_2 evolving from the root zone. Only roots which had grown into the headspace slightly increased the headspace CO_2 concentration. Those plantlets were excluded from experiments. As stomatal apertures of illuminated plantlets at high air humidity stay open when the CO_2 concentration is increased the headspace CO_2 concentration is assumed to equal that of the intercellular CO_2 concentration, i.e. the curves shown in Fig. 3 can be regarded as photosynthesis *vs.* intercellular CO_2 concentration relationships. It is interesting to note that *in vitro*-grown SO 4 known to be more vigorous than Seyval blanc has higher rates of CO_2 assimilation at saturating CO_2 concentration. While the CO_2 compensation points of both varieties are similar (48 and 50 ppm for SO 4 and Seyval blanc, respectively), the carboxylation efficiency and the rate of photosynthesis at CO_2 saturation ("photosynthetic capacity") of SO 4 plants were significantly higher compared to Seyval blanc.

Headspace CO_2 concentrations: Under the usual *in vitro* culture conditions practised in our institute (50-60 µmol quanta·m^{-2}·s^{-1} (Fluora), 26 °C ambient temperature, 16-h light period) using adult plants c_h values ranged from 145 to 155 ppm CO_2 during the light period. Higher light intensities led to a lowering of c_h, lower light intensities to an increase. E.g., at 130 µmol quanta·m^{-2}·s^{-1} c_h was 130 ppm CO_2, while at 43 µmol quanta·m^{-2}·s^{-1} c_h increased to 160 ppm CO_2. Due to the fact that ambient (room) CO_2 concentrations ranged from 400 to 450 ppm CO_2 it is concluded that under the mentioned *in vitro* conditions rates of photosynthesis are limited by an insufficiant CO_2 supply to the headspace.

When air samples of the headspace were taken at the end of a 10-h dark period the CO_2 concentration had increased to very high concentrations (3000 ppm) due to dark respiration. These values decreased rapidly after the onset of the light period to reach constant levels after ca. 1 h (data not shown).

Photorespiration: Rates of photorespiration were calculated from data of experiments with Seyval blanc plantlets in which we increased the CO_2 concentration in the headspace stepwise from 30 to 1200 ppm. Fig. 4 shows that absolute rates of photorespiration decreased with increasing CO_2 supply from 27.9 to 4.7 mg CO_2 · g dry wt^{-1}·h^{-1} while relative rates of photorespiration decreased from 53.6 to 3.3 % of photosynthesis. From this it can easily be deduced that under the usual *in vitro* culture conditions with c_h values ranging from 130 to 150 ppm CO_2 high rates of photorespiration will cause distinct reductions of CO_2 assimilation due to the low CO_2/O_2 ratio at the chloroplasts.

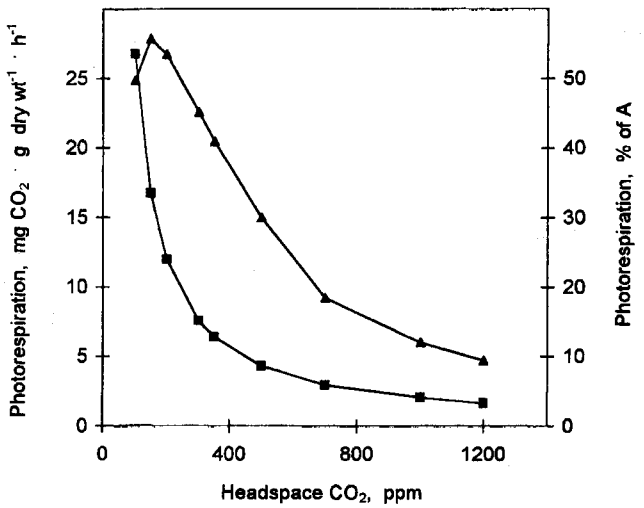

Fig. 4: Absolute (▲) and relative (■) photorespiration rates as a function of CO_2 concentration in the headspace. Variety: Seyval blanc.

This conclusion was further substantiated by a series of experiments with Seyval blanc plantlets: When the normal O_2 concentration of the air (assumed to be ca. 21 %) was lowered to almost zero (replacement of ambient air by N_2) photorespiration was suppressed and CO_2 fixation increased by 37-46 %.

Discussion

There is strong evidence that rates of photosynthesis of *in vitro* plantlets are limited by two major ambient factors: low CO_2 concentration in the headspace of the vessels and low light intensity. The low c_h values are - *inter alia* - a consequence of the severely reduced rates of gas diffusion between the atmosphere and the headspace (FOURNIOUX and BESSIS 1986). This low rate of gas diffusion is necessary to maintain aseptic conditions at high air humidity in the headspace. However, it may lead to an increase of the O_2 concentration in the headspace (RIGHEFFI *et al.* 1993) and thus to an additional shift of the CO_2/O_2 ratio at the chloroplasts towards O_2, thus favoring the oxygenase activity of Rubisco. This explains the high rates of photorespiration under the usual cultural conditions and the beneficial effect of replacing ambient air by nitrogen on photosynthesis. If gas diffusion is further reduced the headspace CO_2 concentration can decline to the CO_2 compensation point as has been shown with *Vitis rupestris* plantlets by FALQUE *et al.* (1991).

In many *in vitro* culture rooms light intensity is another limiting factor of photosynthesis. However, as higher light intensities were shown to lower c_h values the positive effect of an increase of illumination is closely associated with the need to increase gas diffusion between headspace and atmosphere.

The distinct increase of respirational CO_2 during the dark period confirms results of FALQUE *et al.* (1991) who measured CO_2 concentrations of more than 4000 ppm in hermetically sealed vessels containing *V. rupestris* plantlets.

Due to the extreme climatic conditions in the headspace stomata of *in vitro*-grown plantlets are believed to be malformed, malfunctioning or unable to function (e.g. ZIV *et al.* 1987, GHASHGHAIE *et al.* 1992, JONES *et al.* 1993, SALANON *et al.* 1993, MIGUENS *et al.* 1993). On the other hand stomata of *in vitro* plantlets are described to be wide-open at high relative humidity and to close, i.e. to acclimatize when relative humidity is lowered stepwise (BRAINERD and FUCHIGAMI 1982, MARIN and GELLA 1988, JOHANSSON *et al.* 1992, NOVELLO *et al.* 1992, SMITH *et al.* 1992, DENG and DONNELLY 1993). Guard cells of two *in vitro*-grown *V. vinifera* varieties were reported not to develop well under low light and high humidity conditions. As a consequence they opened too wide and did not close completely when treated with abscisic acid or when subjected to low temperature, drought, osmotic stress or darkness (CAO *et al.* 1993). Our results indicate delayed stomatal responses to changes of light intensity and confirm the inability to close completely in the dark. We have shown previously that *in vitro*-grown grapevines, like field-grown vines, have a heterobaric leaf type and that non-uniform stomatal reactions can be expected (DÜRING and STOLL 1996). Thus, it is assumed that incomplete stomatal closure is due to a lowering of the ratio of open to closed stomata. Light-induced stomatal movements may be associated with alterations of the amount of abscisic acid available to the guard cells (COWAN *et al.* 1982). In contrast, changes of CO_2 concentrations in the headspace did not affect stomatal apertures in our experiments. It is speculated that this might be associated with the low availability of abscisic acid in (unstressed) *in vitro* plantlets; abscisic acid has been shown to induce sensitization of stomata to CO_2 (RASCHKE 1975).

References

BRAINERD, K. E.; FUCHIGAMI, L. H.; 1982: Stomatal functioning of *in vitro* and greenhouse apple leaves in darkness, mannitol, ABA and CO_2. J. Exp. Bot. **33**, 388-392.

CAO, Z. Y.; LI, W.; WANG, X. S.; 1993: Effect of ABA and environmental factors on the degree of stomatal opening in grape leaf in test tube. Acta Phytophysiol. Sin. **19**, 371-378.

CHAGVARDIEFF, P.; PEAN, M.; CARRIER, P.; DIMON, B.; 1990: Oxygen exchange capacities in a photoautotrophic bath culture of *Euphorbia characias* cell suspension. Plant Physiol. Biochem. **28**, 231-238.

COWAN, I. R.; RAVEN, J. A.; HARTUNG, W.; FARQUHAR, G. D.; 1982: A possible rate for abscisic acid in coupling stomatal conductance and photosynthetic carbon metabolism in leaves. Austral. J. Plant Physiol. **9**, 489-498.

DENG, R.; DONNELLY, D. J.; 1993: *In vitro* hardening of red raspberry through CO_2 enrichment and relative humidity reduction on sugar-free medium. Can. J. Plant Sci. **73**, 1105-1113.

DÜRING, H.; 1988: CO_2 assimilation and photorespiration of grapevine leaves: Responses to light and drought. Vitis **27**, 199-208.

- -; STOLL, M.; 1996: Stomatal patchiness of grapevine leaves. I. Estimation of non-uniform stomatal apertures by a new infiltration technique. Vitis **35**, 65-68.

FALQUE, M.; COMPAN, D.; GALZY, R.; 1991: A method for the study of CO_2 exchange in *in vitro* cultured *Vitis rupestris* plantlets. Plant Cell Tissue Organ Cult. **27**, 175-181.

FOURNIOUX, J. C.; BESSIS, R.; 1986: Influence de la teneur en gaz carbonique sur la morphogenèse de la vigne en culture *in vitro*. Can. J. Bot. **64**, 2608-2616.

GHASHGHAIE, J.; BRENCKMANN, F.; SAUGIER, B.; 1992: Water relations and growth of rose plants cultured *in vitro* under various relative humidities. Plant Cell, Tissue. Org. Cult. **30**, 51-57.

HARST, M.; 1995: Development of a regeneration protocol for high frequency somatic embryogenesis from explants of the grapevines (*Vitis* spp.). Vitis **34**, 27-29.

JOHANSSON, M.; KRONESTEDT-ROBARDS, E. C.; ROBARDS, A. W.; 1992: Rose leaf structure in relation to different stages of micropropagation. Protoplasma **166**, 165-176.

JONES, N. B.; DRENNAN, P. M.; STADEN, J., VAN; 1993: Leaf anatomy, chloroplast organization and photosynthetic rate of hyperhydric *Eucalyptus saligna* Sm. material. S. Afr. J. Bot. **59**, 551-555.

KOZAI, T.; OKI, H.; FUJIWARA, K.; 1990: Photosynthetic characteristics of *Cymbidium* plantlets *in vitro*. Plant Cell Tissue Org. Cult. **22**, 205-211.

LIMA DA SILVA, A.; HARISCAIN, P.; OLLAT, N.; DOAZAN, J. P.; 1995: Photosynthetic abilities of *Vitis* plantlets (rootstock var. Gravesac) cultivated *in vitro* under different carbon nutrition conditions. In: CARRE, E.; CHAGVARDIEFF, P. (Eds.): Proc. Intern. Symp. Ecophysiology and Photosynthesis of *in vitro* cultures, 203-204. CEA, Cadarache, France.

- -; - -; - -; - -; 1996: Estimation de la capacité photoautotrophique de vitroplants de porte-greffe de vigne (var. Gravesac): mise au point d'un système de mesure de la photosynthèse nette de vitroplants. Vitis **35**, 73-78.

LINSMAIER, E. M; SKOOG, F.; 1965: Organic growth factor requirements of tobacco tissue culture. Physiol. Plant **18**, 120-127.

MARIN, J. A.; GELLA, R.; HERRERO, M.; 1988: Stomatal structure and functioning as a response to environmental changes in acclimatized micropropagated *Prunus cerasus* L. Ann. Bot. **62**, 663-670.

MIGUENS, F. C.; LOURO, R. P.; MACHADO, R. D.; 1993: A scanning electron microscope study of normal and vitrified leaves from *Datura insignis* plantlets cultured *in vitro*. Plant Cell, Tissue Org. Cult. **32**, 109-113.

NOVELLO, V.; GRIBAUDO, I.; ROBERTS, A. V.; 1992: Effects of paclobutrazol and reduced humidity on stomatal conductance of micropropagated grapevines. Acta Horticulturae **319**, 65-70.

RASCHKE, K.; 1975: Stomatal action. Ann. Rev. Plant Physiol. **26**, 309-340.

RIGHETTI, B.; MAGNANINI, E.; ROSSI, F.; 1993: Photosynthetic carbon dioxide uptake and oxygen accumulation during *in vitro* culture of *Actinidia deliciosa* cv. Tomuri. Environ. Exp. Bot. **33**, 523-528.

SALLANON, H.; TORT, M.; COUDRET, A.; 1993: The ultrastructure of micropropagated and greenhouse rose plant stomata. Plant Cell Tissue Org. Cult. **32**, 227-233.

SHARKEY, T. D.; 1988: Estimating the rate of photorespiration in leaves. Physiol. Plant. **73**, 147-152.

SMITH, E. F.; GRIBAUDO, I.; ROBERTS, A. V.; MOTTLEY, J.; 1992: Paclobutrazol and reduced humidity improve resistance to wilting of micropropagated grapevine. Hortscience **27**, 111-113.

WIDHOLM, J. M.; 1995: Photosynthetic plant cell cultures: An overview. In: CARRE, E.; CHAGVARDIEFF, P. (Eds.): Proc. Intern. Symp. Ecophysiology and Photosynthesis of *in vitro* cultures, 15-24. CEA, Cadarache, France.

Received September 9, 1996