Acta Bot. Neerl. 41(4), December 1992, p. 417–423

Ecological and physiological aspects of tissue-cultured plants

P. C. DEBERGH*, J. DE MEESTER, J. DE RIEK, S. GILLIS‡ and J. VAN HUYLENBROECK‡

Laboratory of Horticulture and ‡IWONL centre, University Gent, Coupure 653, 9000 Gent, Belgium

SUMMARY

Tissue-cultured plants are different from normal plants because of the environmental conditions in a tissue-culture container. Together with the water retention capacity (WRC) of the headspace in a container, the gas composition is responsible for a divergent physiological behaviour. By controlling the WRC of the headspace the physiology and anatomy of tissue-cultured plants can be improved to resemble normal plants.

During culture *in vitro* under our conditions, the CO_2-level showed a typical circadian shift depending upon illumination, but was always higher than the normal atmospheric concentration. Carbon accumulating in tissue-cultured plants *in vitro* originated both from headspace-CO_2 and sucrose in the culture medium. The evolution of photosynthesis during the acclimatization process allowed the determination of the condition of the plant and indicated the occurrence of stress after planting and after transfer from weaning to normal conditions.

Key-words: acclimatization, autofluorescence, ecology, photosynthesis, physiology, tissue culture.

INTRODUCTION

Normal looking tissue-cultured plants (TC-plants) are quite often anatomically and physiologically aberrant due to the ambient conditions *in vitro*.

For tissue culture closed containers are used to avoid infections. As a consequence, the composition of the headspace in those containers differs from the ambient atmosphere of the culture room. The most obvious headspace components that are influenced by this situation, are the relative humidity and the CO_2-content. Both will be discussed in the present article.

MATERIALS AND METHODS

CO_2-evolution in tissue-culture containers

Plant material and culture conditions. Rosa multiflora L. cv. Montsé was micropropagated by monthly subcultures in Meli jars (370 ml) on 100 ml Stage II medium (Cappelades

*To whom correspondence should be addressed.

1989) with 3% sucrose (10 shoots per jar). In the upper edge of the jar a small nick was made to improve gas exchange with the environment. The jars were closed by a polycarbonate screw-on lid, with a serum cap inserted for headspace sampling during the experiments. The jar was wrapped in a stretch film to minimize water loss. Culture conditions were: temperature $23 \pm 2°C$; photosynthetic active radiation (PAR) ± 35 µmol m^{-2} s^{-1}; 16 h light (fluorescent tubes type Osram L36W/31 Warnton Lumilux Warm White), 8 h darkness. Relative humidity in the jar was not controlled.

CO_2-evolution analysis by gas chromatography. CO_2 was measured by gas chromatography. Using gas-tight syringes, 1 ml samples were taken and injected into a Delsi Nermag IGC11M gas chromatograph, equipped with a thermal conductivity detector (TCD). The type of column was a dual-phase GC-column (CTR II, BIORAD RSL N.V., Eke, Belgium) consisting of an inner column (packed with Porapack to separate air, methane and carbon dioxide) and an outer (packed with a molecular sieve 50^{-1} nm to separate oxygen, nitrogen, methane and carbon monoxide). Operating conditions were: column temperature: 30°C; flow rate of the carrier gas (He): 25 ml min^{-1}; current through the conductor: 180 mA. Retention times and integration values of the peaks were determined with a Enica 31 recorder-integrator linked to a personal computer and equipped with appropriate software. An appropriate standard solution (BIORAD RSL N.V., Gent, Belgium) was provided with the CTR II column. CO_2 was expressed in µl l^{-1} (ppm). The CO_2-evolution was observed over 24 h, sampling five jars each hour. This was done four times during the whole culture period, taking five new jars for each sampling time.

Chlorophyll-a fluorescence kinetics

The intensity of chlorophyll fluorescence from leaves of *Gerbera jamesonii* plants was measured in a non-destructive way with a fluorometer model SF-30 (Richard Branker Research Ltd, Ottawa, Canada). The fluorometer has a cylindrical sensing probe with a 20 mm diameter opening on the lower side which contains a light-emitting diode, providing illumination centred around 670 nm, and a photo-diode sensor which detects light of a wavelength longer than 710 nm.

Before measuring chlorophyll fluorescence, plants were kept in the dark for 30 min. After this dark adaptation the upper surface of a full expanded leaf was illuminated with a light intensity of 3 W m^{-2} (1 W = 5·59 µmol s^{-1}) during 150 s. Fluorescence induction curves were stored in a microprocessor unit and the fluorescence parameters I (intermediary level), P (peak level) and T (terminal level) from the Kautsky curve were calculated (Govindjee *et al.* 1986).

RESULTS AND DISCUSSION

Control of water retention capacity

The high relative humidity is the major driving force behind the phenomenon called 'vitrification'. It has been suggested (Debergh *et al.* 1992) that the use of this term should be reconsidered as it is also used in cryobiology. The term 'hyperhydricity' has been proposed as an alternative.

The confined conditions combined with the heating of the bottom of the container by the lights installed on the lower level, are responsible for the high relative humidity in a container. This situation creates a limited difference in water retention capacity between the stomatal cavity and the headspace and is therefore the major cause of all kinds of both

subtle and obvious abnormalities related to hyperhydricity (Ziv 1991). Different methods have been proposed to lower the relative humidity: increasing the agar concentration (Debergh *et al.* 1981); using an agar with a higher gel strength (Debergh 1983); increasing the aeration of the container (e.g. Dillen & Buysens 1989); overlaying the medium with a paraffin layer (Wardle *et al.* 1983); and increasing the light intensity (L. J. Maene, pers. comm.). All these methods lower the relative humidity but do not allow control. Problems related to high salt concentrations can occur especially when water vapour can escape from the container. The only practical method for controlling the relative humidity is bottom cooling: water is maintained in the container and the required relative humidity is installed at will (Maene & Debergh 1987; Vanderschaeghe & Debergh 1987). Bottom cooling yields more 'normal' plants. This has been exemplified with *Rosa*: hydathodes are less numerous, stomata are more functional (Capellades *et al.* 1990a) and water retention capacity of leaves is improved (Capellades 1989).

CO_2-evolution in tissue culture containers

At present there is a lot of interest in the photo-autotrophic micropropagation system as proposed by Kozai *et al.* (Kozai 1991 and references mentioned herein). The general belief is that at transition from *in vitro* to *in vivo* conditions plants should be as autotrophic as possible to overcome the transplantation shock with least difficulties. It is also believed that CO_2 is a limiting factor during the *in vitro* stages. Under our experimental conditions neither of these statements have been confirmed.

Capellades *et al.* (1990b) have shown that the least photo-autotrophic plants of *Rosa multiflora* give the best results (time and success rate) during the acclimatization stage. In the course of micropropagation, the CO_2-concentration always remained higher than the normal atmospheric concentration of 350 ppm ($=350\ \mu l\,l^{-1}$) (Fig. 1) and showed a typical day–night pattern (we used 30 $\mu mol\ m^{-2}\ s^{-1}$, Kozai (1991) used approximately 250 $\mu mol\ m^{-2}\ s^{-1}$). These results have been confirmed for other different species, namely *Asparagus officinalis, Cordyline terminalis, Prunus* spp. and *Sequoiadendron.*

Photosynthesis in vitro

The carbon metabolism of micropropagated roses was quantified by using two labelled substrates: ^{14}C-sucrose and ^{14}C-CO_2. It was shown that growth caused by CO_2-fixation, varies between 25 and 60%, depending on the cultivar (De Riek *et al.* 1991). An example of the distribution over the different fractions is shown in Fig. 2.

The evolution of photosynthesis during acclimatization

Chlorophyll-a fluorescence kinetics was used to follow the evolution of photosynthesis of micropropagated *Rosa multiflora* (Capellades *et al.* 1990b) and *Gerbera jamesonii* during the acclimatization stage. The (P−I)/I ratio of the Kautsky curve revealed that their changes agreed with those of leaf net photosynthesis (Capellades *et al.* 1991). From the results of Capellades *et al.* (1991) it is obvious that the least autotrophic plants accumulated most starch in their chloroplasts and are best suited for acclimatization under the experimental conditions proposed.

An example of the evolution of the (P−I)/I ratio for *Gerbera jamesonii* is presented in Fig. 3. This figure indicates the successions of stress. Immediately after transplanting to greenhouse conditions the (P−I)/I ratio for all treatments increased (this reveals an increase in photosynthesis), but after 1 week a significant decrease occurred. This decrease probably coincided with the depletion of the starch stock in the chloroplasts. Then, the

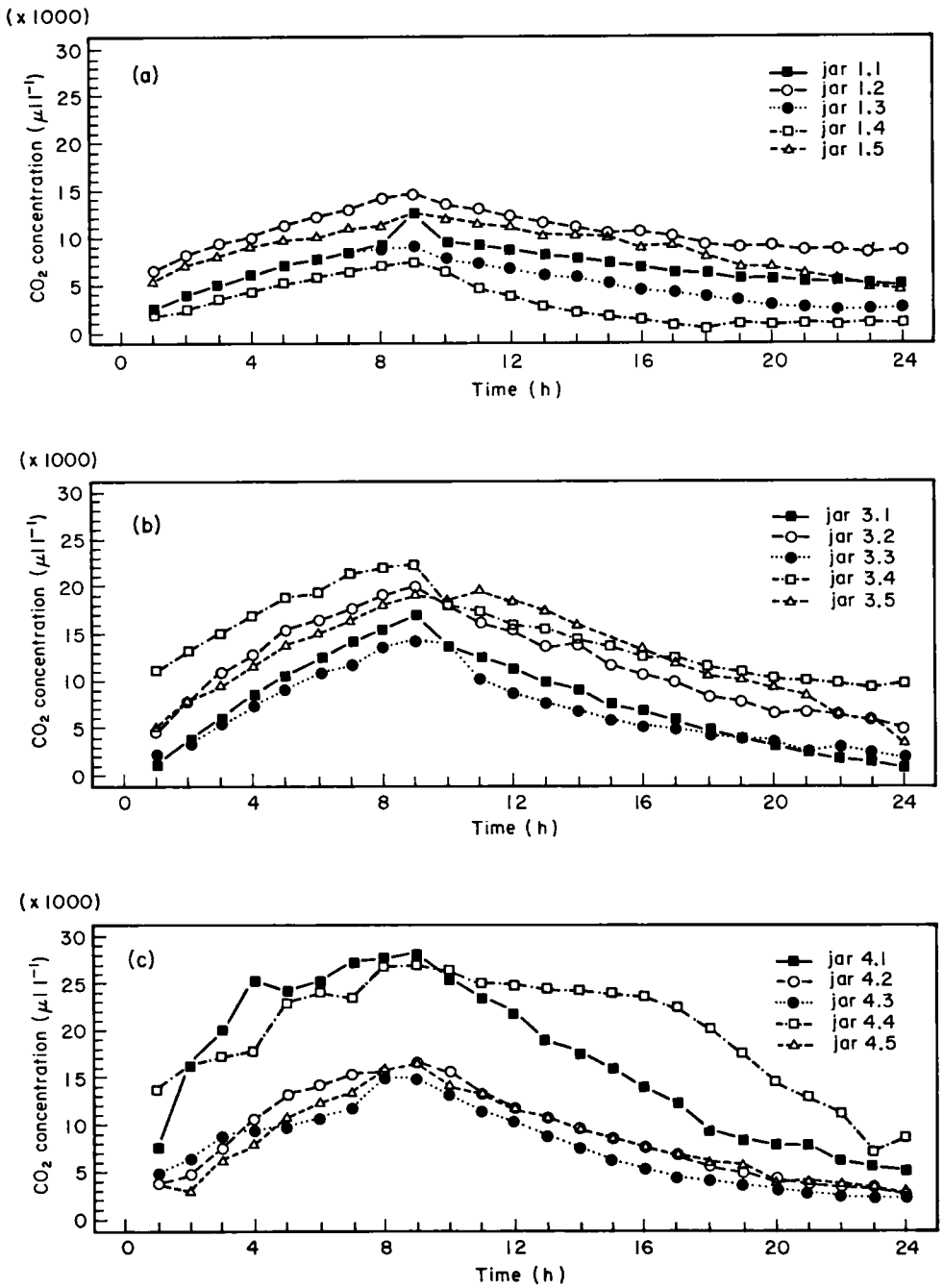

Fig. 1. CO_2-evolution in micropropagated *Rosa multiflora* over 24 h on Day 10 (a), Day 28 (b) and Day 41 (c) of the propagation cycle ($t = 1$–9: dark period; $t = 9$–24: light period; light intensity 30 μmol $m^{-2}\,s^{-1}$).

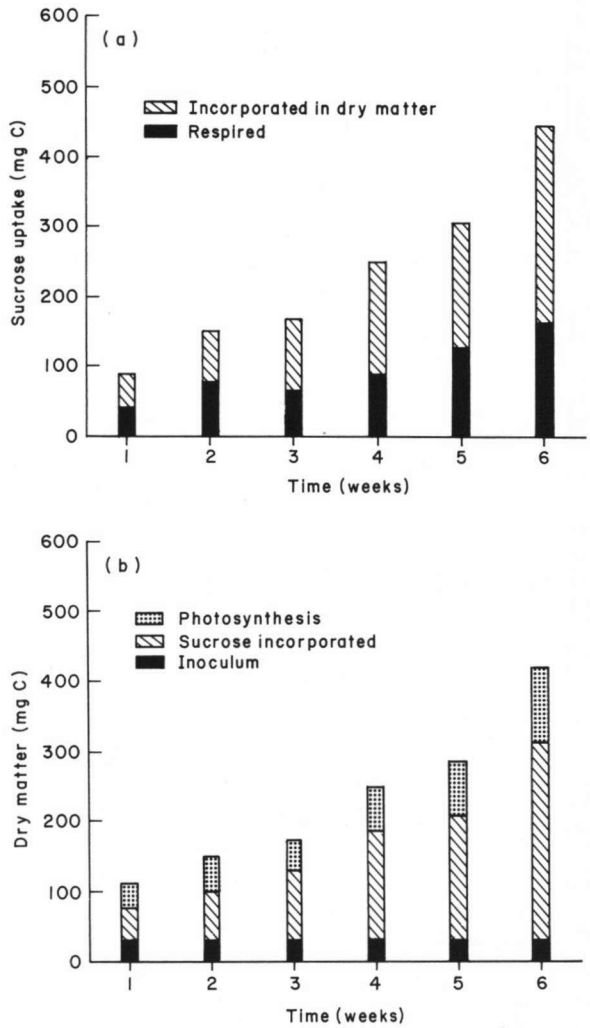

Fig. 2. The evolution of the sucrose uptake (a) and the total dry matter increase (b) of stage-2 cultures of micropropagated *Rosa multiflora*. The repartitioning over the different fractions is presented.

ratio raised again (plants had rooted). When the plants were transplanted from weaning conditions (high relative humidity) to a conventional greenhouse (low relative humidity) at 3 weeks after transplanting from the tissue-culture container the ratio decreased again. After 5 weeks, an average ratio of 0·32 was observed reaching 0·56 after 8 weeks. This indicates the improved photosynthesis.

Figure 3 also illustrates that raising the CO_2-concentration during acclimatization from 450 (Treatments 3 and 4) to 900 ppm (Treatments 1 and 2) hastened the acclimatization process. Moreover the quality of the plants had improved (Fig. 4).

CONCLUSION

The quality of *in vitro* plants can be improved by lowering the humidity in the headspace of the container. During the *in vitro* stages, CO_2 was never in shortage (always higher than

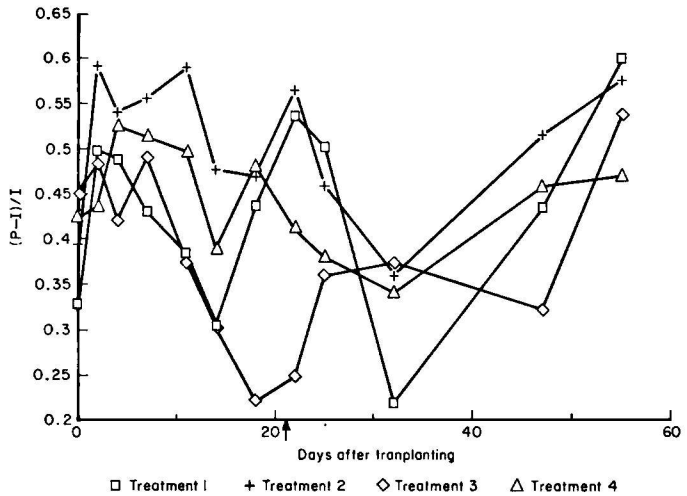

Fig. 3. Evolution of the (P−I)/I ratio during the acclimatization of *Gerbera jamesonii*. Treatment 1: CO_2 900 ppm, PAR 95 µmol $m^{-2} s^{-1}$; Treatment 2: CO_2 900 ppm, PAR 52 µmol $m^{-2} s^{-1}$; Treatment 3: CO_2 450 ppm, PAR 95 µmol $m^{-2} s^{-1}$; Treatment 4: CO_2 450 ppm, PAR 52 µmol $m^{-2} s^{-1}$ (the arrow indicates the transfer from weaning to normal conditions).

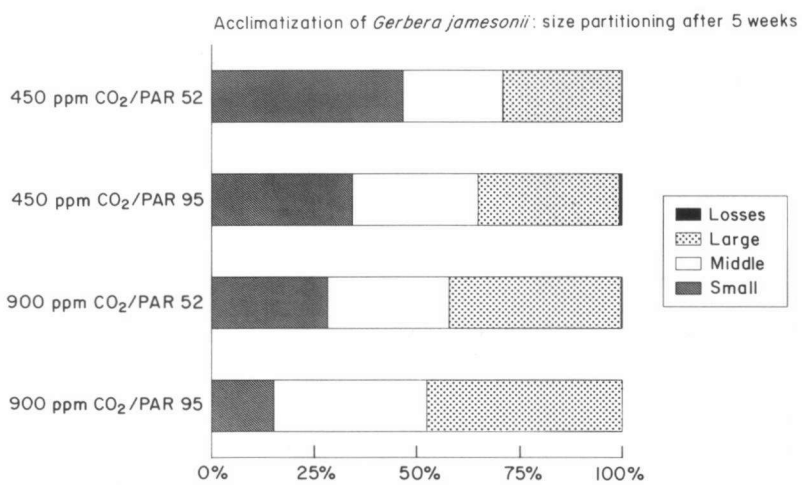

Fig. 4. Size repartition of micropropagated *Gerbera jamesonii*. For the different treatments see Fig. 3.

350 µl l^{-1}), and its concentration followed a typical night–day curve. A considerable part (between 25 and 50%, De Riek *et al.* 1991) of the growth in stage-2 cultures was caused by CO_2-fixation.

During the acclimatization process, the least photo-autotrophic shoots behaved the best. Chlorophyll-a fluorescence enabled the evolution of the photosynthetic process during the acclimatization to be traced. High CO_2-concentration (900 ppm) favoured photosynthesis and quality of the plants.

REFERENCES

Capellades, M. (1989): *Histological and ecophysiological study of the changes occurring during the acclimatization of in vitro cultured roses.* PhD dissertation, State University Gent.

Capellades, M., Fontarnu, R., Carulla, C. & Debergh, P. (1990a): Environment influences anatomy of stomata and epidermal cells in tissue cultured *Rosa multiflora*. *J. Am. Soc. Hort. Sci.* **115:** 141–145.

Capellades, M., Lemeur, R. & Debergh, P.C. (1990b): Kinetics of chlorophyll fluorescence in micropropagated rose shootlets. *Photosynthetica* **24:** 190–193.

Capellades, M., Lemeur, R. & Debergh, P.C. (1991): Effects of sucrose on starch accumulation and rate of photosynthesis in *Rosa* cultured *in vitro*. *Plant Cell Tiss. Organ Cult.* **25:** 21–26.

Debergh, P.C. (1983): Effects of agar brand and concentration on the tissue culture medium. *Physiol. Plant.* **59:** 270–276.

Debergh, P.C., Aitken-Christie, J., Cohen, D., Grout, B., Von Arnold, S., Zimmerman, R. & Ziv, M. (1992): Reconsideration of the term 'vitrification' as used in micropropagation. *Plant Cell Tiss. Organ Cult.* **30:** 135–140.

Debergh, P.C., Harbaoui, Y. & Lemeur, R. (1981): Mass propagation of globe artichoke: evaluation of different hypothesis to overcome vitrification with special reference to water potential. *Physiol. Plant.* **53:** 181–187.

De Riek, J., Van Cleemput, O. & Debergh, P.C. (1991): Carbon metabolism of micropropagated *Rosa multiflora* L. *In vitro Cell. Dev. Biol.* **27P:** 57–63.

Dillen, W. & Buysens, S. (1989): A simple technique to overcome vitrification in *Gypsophylla paniculata*. *Plant Cell Tiss. Organ Cult.* **19:** 181–188.

Govindjee, Amesz, J. & Fork, D.C. (1986): *Light emission by plants and bacteria.* Academic Press Inc., New York.

Kozai, T. (1991): Micropropagation under photoautotrophic conditions. In: Debergh P.C. and Zimmerman, R.H. *Micropropagation: Technology and Application.* 447–469. Kluwer Academic Publ., Dordrecht.

Maene, L.J. & Debergh, P.C. (1987): Optimalization of the transfer of tissue cultured shoots to *in vivo* conditions. *Acta Hort.* **212:** 335–348.

Vanderschaeghe, A.M. & Debergh, P.C. (1987): Technical aspects of the control of the relative humidity in tissue culture containers. *Med. Fac. Landbouww. Gent* **52:** 1429–1437.

Wardle, K., Dobbs, E.B. & Short, K.C. (1983): *In vitro* acclimatization of aseptically cultured plantlets to humidity. *J. Am. Soc. Hort. Sci.* **108:** 386–389.

Ziv, M. (1991): Vitrification: morphological and physiological disorders of *in vitro* plants. In: Debergh P.C. and Zimmerman R.H. (eds) *Micropropagation: Technology and Application.* 45–69. Kluwer Academic Publ., Dordrecht.