Relationship between tissue growth, CO_2 level and tendril formation during *in vitro* culture of grape (*Vitis vinifera* L.)[1]

by

P. Thomas

Division of Biotechnology, Indian Institute of Horticultural Research, Bangalore, India

S u m m a r y : *In vitro* cultures of grapevine cultivar Arka Neelamani growing in Murashige and Skoog (1962) medium containing 3 % sucrose and incubated under 16 h light (30-40 $\mu mol \cdot m^{-2} \cdot s^{-1}$) per day showed high CO_2 levels (600-11900 ppm) at the end of the dark period and a significant decline during the light period (330-980 ppm) indicating photosynthetic activity and photomixotrophic growth characteristic. The CO_2 level at the end of a 8-h-dark period was shown to be related to the total tissue weight (wt) per culture vessel while that during the light period was governed by the root to shoot fresh wt ratio suggesting a notable contribution by roots and inadequate light supply for full CO_2 utilization. Plant growth was improved by higher light intensities (40-50 $\mu mol^\circ m^{-2\circ} s^{-1}$). Tendril production, a characteristic of adult plants, was common during the first year of culture but showed a gradual decline by the second and third year suggesting a gradual rejuvenation effect *in vitro*. Transformation of tendril, an organ homologous to inflorescence, to leafy structure in some plants and better rooting with more subcultures *in vitro* further supported the rejuvenation hypothesis. In general, tendril production was more common in vessels with higher CO_2 levels. Plants established *ex vitro* invariably showed tendril production within 1-3 months indicating that the *in vitro* rejuvenation effect was not transmitted to the field.

K e y   w o r d s : CO_2, light, grapes, in vitro, micropropagation, photosynthesis, rejuvenation, tendril.

Introduction

One of the limitations in grape micropropagation is the return of cultures to juvenile morphology after several subcultures that may even be transmitted to the field (Grenan 1992; Fournioux and Bessis 1993). Characteristics of rejuvenation include a decrease or even disappearance of tendrils and a shift to spiral phyllotaxy (Mullins *et al.* 1979; Fournioux and Bessis 1993). Adult *V. vinifera* vines show distichous phyllotaxy and a tendril opposite to leaf at two out of three nodes in a repetitive manner, usually designated as N0-N1-N2 arrangement (Bouard 1966; Weaver 1976; Mullins *et al.* 1979). Fournioux and Bessis (1993) were able to improve normal pattern of tendril production in *V. vinifera* cultures *in vitro* through incubation in CO_2 enriched (1200 ppm) environment. Studies undertaken in this laboratory with *V. vinifera* cv. Arka Neelamani showed tendril production under normal incubating conditions even after 3 years of *in vitro* culture. Therefore, it was suggested to study the CO_2 level in these cultures. Most of the *in vitro* studies involving CO_2 focus on the effect of CO_2 enrichment on photosynthesis aiming at photoautotrophic micropropagation (reviews: Kozai 1991 a, b; Kozai *et al.* 1997). Some effort has been made in *Vitis* sp. to study the CO_2 levels and rates of photosynthesis:respiration (Falque *et al.* 1991; Galzy and Compan 1992; Düring and Harst 1996; Lima da Silva *et al.* 1996; Iacono and Martinelli 1998) and these studies have suggested significant effects of genotype and culture conditions warranting more research under different incubating conditions. The present studies were

undertaken to monitor the CO_2 level during the periods of light and darkness in grape cultures with reference to growth and to relate tendril production to *in vitro* CO_2 levels.

Material and Methods

Studies were carried out using grapevine (*Vitis vinifera* L.) variety Arka Neelamani. *In vitro* cultures were established on Murashige and Skoog (1962) medium containing 3 % sucrose, 0.25 % phytagel (Sigma Co., USA), 1 μM IAA and 0.1 μM GA_3 and were further micropropagated at 1-3-month intervals using shoot tip and nodal microcuttings (Thomas 1997). The experimental unit comprised of glass culture bottles (12 cm height, 6.5 cm wide, 5 cm mouth) planted with 4 microcuttings in 50 ml medium and closed with semi-transparent tight polycarbonate screw caps (Thomas 1997) unless specified otherwise. The cultures were incubated at 26 ± 2 °C and a 16-h-light period (30-40 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at culture level provided by cool fluorescent tubes. Cultures were observed for tendril production regularly from the time of *in vitro* establishment up to 3 years as well as after *ex vitro* establishment employing sachet technique (Ravindra and Thomas 1995; Thomas 1998 a). Measurements of CO_2 levels were made in the second and third years. The timing of the light/dark cycles was suitably modified to carry out the determinations during day time between 10-16 h.

In order to assay CO_2 accumulation in culture vessels in relation to light and dark periods, one culture each originat-

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ing from shoot tip and nodal cuttings was selected at one or two months after sub-culturing. The cultures were set to a 16-h-light period starting at 10 a.m. initially. A small hole (0.5 mm) had been drilled in the plastic cap of the bottles and this hole was covered with two layers of cellotape. Using a syringe, 1 ml air sample was drawn from each culture bottle at the beginning of the light period and every 2 h thereafter up to 6 h. Similar samples were drawn from the same vessels at the end of the light period after resetting the dark period to commence at 10 a.m. The CO_2 level was determined using a GC (5890 Series II, Hewlett Packard, USA). To test the gas permeability of culture vessels, pure CO_2 was injected into 4 bottles containing 50 ml medium but without plants. CO_2 level was monitored at 1 h and 24 h thereafter and the diffusion per hour was determined.

The CO_2 concentration in the atmosphere of cultures at the end of dark period was determined using 42 culture vessels and this was done in 5 batches. These vessels included one-month-old cultures after previous subculturing and had varying growth by using different populations per vessel (2 or 4 microcuttings per bottle), types of microcuttings (shoot tip, leafy nodal or leafless cuttings) or medium containing dextrose (0, 1 or 2 %) with 3 % sucrose, which are known to influence root and shoot growth *in vitro* (THOMAS 1997 and 1998 b). In addition, three incubation periods (1, 2 or 3 months using 4 leafy nodal cuttings per vessel) were included. There were 3-6 culture vessels in each of the above category. One air sample (1 ml) was drawn per bottle and the CO_2 level was determined by GC. The data from different categories were pooled for statistical analysis. The fresh weight (wt) of shoot and root tissue in each culture vessel was determined and the correlation coefficient (r) between CO_2 level and tissue wt or root/shoot wt ratio was estimated (FRIEDMAN 1972).

CO_2 in cultures during light period was monitored in 48 vessels using a portable infra red gas analyser (IRGA) (*LI*- 6200 Photosynthesis unit, LI-COR Inc., Lincoln, USA). (IRGA had an upper CO_2 detection limit of 1800 ppm and therefore it could not be used for determinations at the end of dark period). The observations were made in the culture room 9-10 h after the onset of a light period in 3 batches consisting of cultures differing in growth. The data from different categories were pooled for statistical analysis.

Results and Discussion

The microcuttings developed single shoots with 4-6 nodes and rooting within 1 month of culturing. Production of small often bifid tendrils agreeing to the N0-N1-N2 pattern (Fig. 1 a) was common during the first year in culture. Most of the plantlets showed only one set of tendrils and rarely 2-3 sets when observed 2-3 months after culturing. The upward growth was restricted by the caps leading to coiling under the caps (THOMAS 1997). Tendril production was influenced by factors including the type of cutting used for subculturing, population in a vessel, period after previous subculturing and time from culture initiation. For
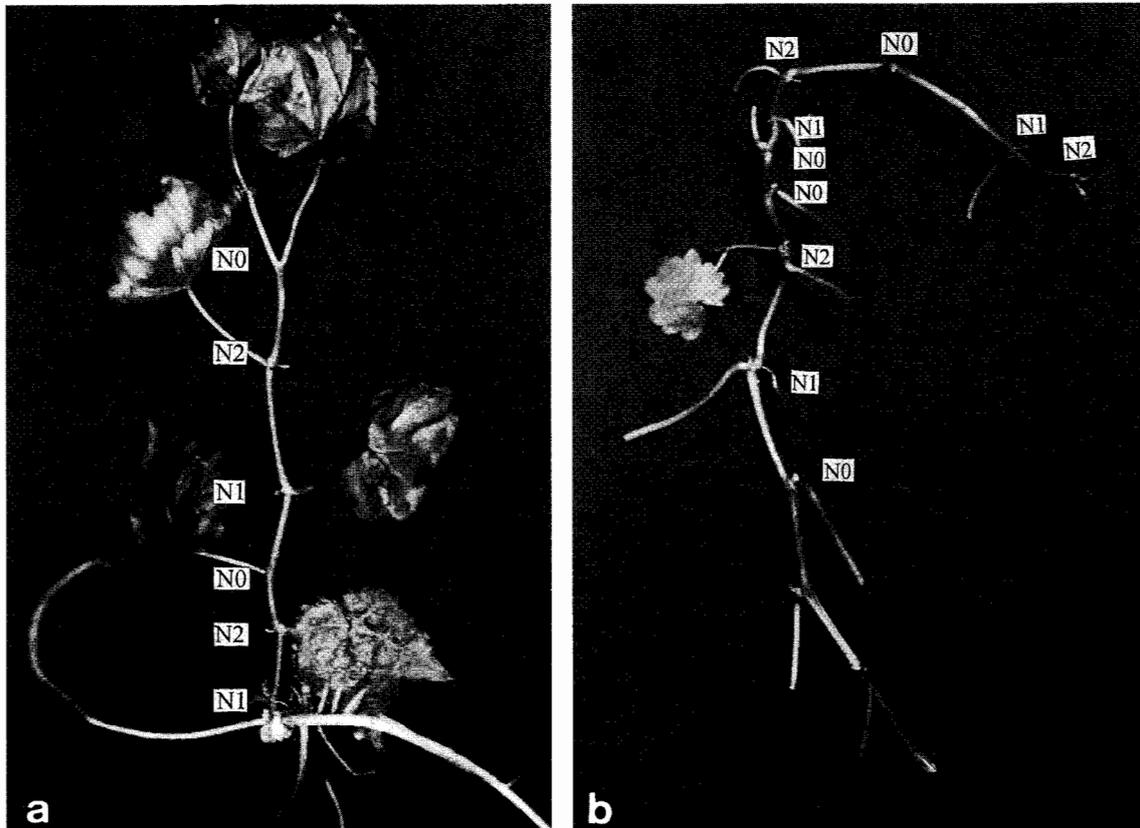

Fig. 1: (a) Normal pattern (N0-N1-N2) of tendril formation in a 6-week-old plantlet derived from shoot tip microcutting, and (b) deviation from normal pattern of tendril formation in a 9-week-old plantlet with one branch of a bifid tendril transformed to a leafy structure. (Tendril arrangement is according to BOUARD (1966): N0 = tendril lacking node; N1 and N2 = tendril bearing nodes).

instance, shoot tip cultures showed tendril production from earlier nodes (1st to 4th from the base) while nodal cuttings exhibited tendrils at later nodes (5th to 10th). Not all plants in a vessel showed tendrils. Plants showing tendril formation one month after subculturing dropped from 78 % during the first year to 16 % by the end of the third year and this became erratic too. Deviation from normal pattern included two successive nodes without tendrils (N1-N0-N0-N1 or N2-N0-N0-N1) or a single tendril bearing node between two others without tendril (N0-N1-N0) as observed by FOURNIOUX and BESSIS (1993). Seldom cultures showed the transformation of a branch of the bifid tendril to leafy structure after 2-3 years in culture (Fig. 1 b). *In vivo*, tendril and inflorescence are homologous organs and tendril considered is a slightly differentiated inflorescence (SRINIVASAN and MULLINS 1978; MULLINS *et al.* 1979). Thus, the transformation of tendril to leafy structure is an indication of rejuvenation *in vitro*. During the initial subcultures, cv. Arka Neelamani exhibited poor rooting and root vigour (THOMAS 1997) but showed a substantial improvement with repeated subcultures. Improved rooting with more subcultures has been observed in other crops too (SRIKANDARAJAH *et al.* 1982; RUGINI and VERMA 1993) and is probably an indication of rejuvenation in culture (NÉMETH 1986) or adaptation to *in vitro* environment (THOMAS 1997).

The culture vessels without plants allowed a CO_2 diffusion rate of 250-260 ppm h^{-1} over a 24-h period when CO_2 (1.7-3.7 %) was injected. In vessels with plants CO_2 level declined below the ambient level within 2-4 h after the onset of the light period irrespective of the level at the end of the previous dark period and reached a slightly higher value after 6 h (Fig. 2 a) suggesting an initial increase of photosynthesis with the supply of light. The value at the end of the light period was comparable to that after 6 h in light and there was a steady increase of the CO_2 level in the dark (Fig. 2 b). CO_2 levels at the end of the dark period and their rate of decline during the light period varied with the age of culture and the type of cuttings used for culturing reflecting a relation to tissue growth in the vessel.

The CO_2 level in the atmosphere of culture vessels at the end of the dark period varied from 600 to 11900 ppm in 41 out of 42 cultures studied and this was found to be influenced by the total tissue wt in a culture vessel (Fig. 3). A highly significant correlation existed between the CO_2 level

in the vessels and total tissue wt in all 5 individual batches and in the pooled analysis involving all cultures ($r = 0.831$). Thus, the variation of CO_2 from vessel to vessel could be explained by the total tissue wt in vessels. One out of 42 cultures displayed very high CO_2 (19.9 %). This particular culture had very vigorous root growth but poor shoot growth, the latter accounting for less than 10 % of the total tissue wt. The roots were thick and leaves appeared leathery and dark green. This culture did not show any latent bacteria when tested on a medium according to VISS *et al.* (1991). In other cultures, the root/shoot wt ratio generally varied from 0.5 to 2.0.

The CO_2 in 48 culture vessels recorded after 9-10 h in the light varied from 330 to 980 ppm at an ambient level of 470-480 ppm. The CO_2 level in vessels was significantly correlated to root/shoot wt ratio ($r = 0.705$). Leafy nodal cuttings with high root growth exhibited highest CO_2 values while defoliated nodal cuttings with poor root growth (THOMAS 1988 b) showed very low CO_2 values. As the root/shoot wt ratio increased, the cultures showed an increase in CO_2 concentration (Table). This indicates that respiring roots contribute significantly to headspace CO_2. The decline in CO_2 in the light period underlies the photosynthetic ability (FALQUE *et al.* 1991; KOZAI, 1991 a, b; ROSS-KARSTENS *et al.* 1996) but the higher values above the ambient CO_2 level in most of the cultures hints at inadequate light intensity for its full utilization. This suggested photomixotrophy to occur (GEORGE 1993; KOZAI 1991 b; KOZAI *et al.* 1997) in these cultures. Thus, the *in vitro* growth could probably be improved either by providing shorter light and dark cycles (4 h light/2 h darkness) (MORINI *et al.* 1990) or by increasing light intensity (GEORGE 1993). Increasing light intensity to 40-50 μmol°m^{-2}°s^{-1} by reducing the distance from the light source to the vessel cap level led to a clear improvement in growth (Fig. 4 a, b) and green colour of plantlets. Other studies which report very low CO_2 levels during the light period (FALQUE *et al.* 1991; KOZAI 1991 a, b; DÜRING and HARST 1996; ROSS-KARSTENS *et al.* 1996; KOZAI *et al.* 1997) have utilised higher light intensities (>100 μmol°m^{-2}°s^{-1}) compared to the present study indicating that the CO_2 level during the light period is influenced by light intensity (DÜRING and HARST 1996).

In general, cultures with high CO_2 levels at the end of the dark period tended to show tendril formation more fre-
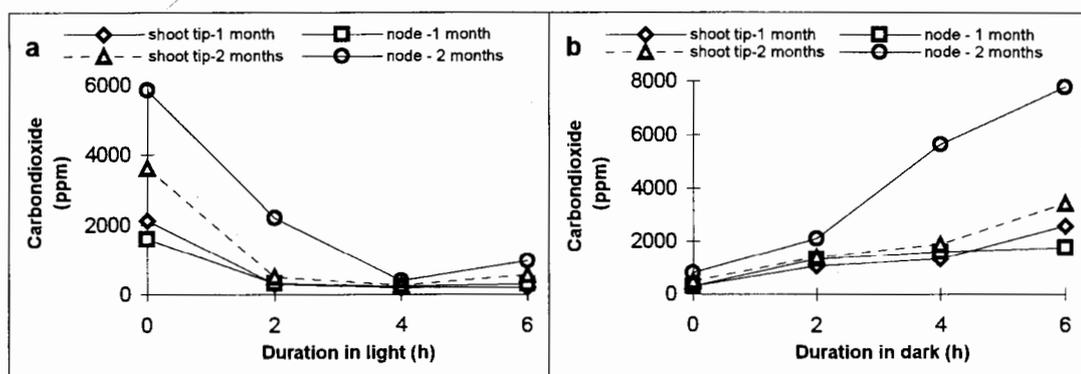

Fig. 2: CO_2 levels in 4 culture vessels differing in type of cuttings (shoot tip or leafy node) used for planting and time after culturing (1 or 2 months) (**a**) during the transition from dark period to light and (**b**) from light to dark period.
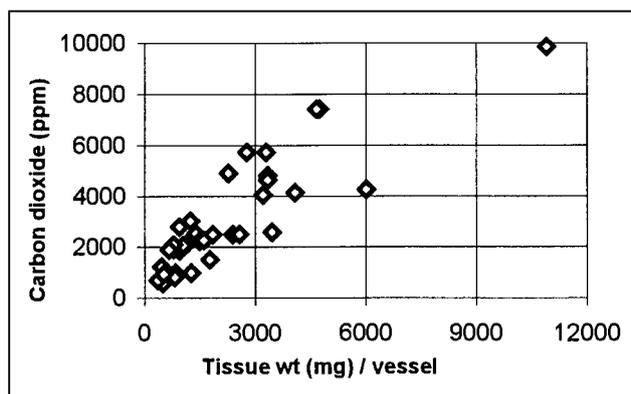
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Fig. 3: Scatter diagram showing CO_2 level in culture vessels at the end of a 8 h dark incubation in relation to total tissue weight per vessel.

quently than those with relatively low CO_2. However all the plants in such vessels did not show tendril formation and there was no parallel increase in tendril formation capability with CO_2 levels. There may be an optimum level of CO_2 for stimulation of tendril formation as observed by FOURNIOUX and BESSIS (1993). Moreover, the humidity inside the vessels and aeration might be involved in regulation of tendril formation, too. In a study where the humidity in culture tubes (25 x 20 cm), planted with single cuttings in 15 ml medium, was reduced by use of cellulose nitrate filter on the plastic cap, about 50 % of the plantlets (second year in culture) showed tendril formation compared to only 17 % in those

having caps covered with parafilm maintaining 9.5 % higher humidity in the vessel (unpublished results).

About 1-3 months from planting, cultures *ex vitro* showed long, bifid tendrils within 10-15 nodes from the base. It was not possible to confirm the phyllotaxy of the plants while inside the vessels but *ex vitro* the plants showed distichous phyllotaxy. The appearance of normal tendrils and distichous phyllotaxy in plants established *ex vitro* indicated that the rejuvenation effect *in vitro*, if any, would not be transmitted to the field.

Acknowledgements

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Table

Average CO_2 concentration in 48 vessels of grape culture during the light period, root/shoot weight (wt) ratio and ratio of total tissue wt to wt of shoot tissue per vessel

CO_2 range (ppm)	Average CO_2 (ppm)	Frequency	Ratio of root/shoot wt	Ratio of total tissue wt/ shoot tissue wt
300-400	362.0 ± 6.0	7	0.711 ± 0.076	1.66 ± 0.11
401-500	454.0 ± 8.6	12	0.721 ± 0.062	1.72 ± 0.06
501-600	547.0 ± 7.2	13	0.773 ± 0.054	1.77 ± 0.05
601-700	660.2 ± 10.1	6	0.927 ± 0.101	1.92 ± 0.10
701-800	738.4 ± 13.3	5	1.179 ± 0.108	2.08 ± 0.75
801-900	826.8 ± 7.2	4	1.374 ± 0.109	2.37 ± 0.11
901-1000	983	1	1.54	2.5

Correlation coefficient (r) between CO_2 level and ratio of root/shoot wt = 0.966; between CO_2 level and the ratio of total tissue wt/shoot tissue wt per vessel = 0.971.

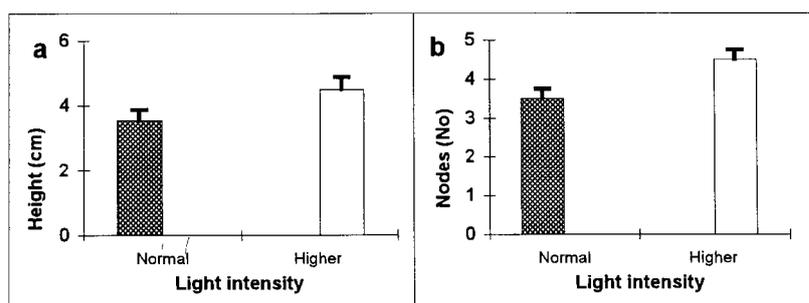

Fig. 4: Plant growth characteristics (a) plant height and (b) number of nodes at normal (30-40 $\mu mol.m^{-2}.s^{-1}$) and higher (40-50 $\mu mol.m^{-2}.s^{-1}$) light intensities.

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