BIOCELL
2003, 27(2): 205-212

ISSN 0327 - 9545
PRINTED IN ARGENTINA

Micropropagation of *Ilex dumosa* (Aquifoliaceae) from nodal segments in a tissue culture system

C. Luna, P. Sansberro*, L. Mroginski and J. Tarragó

Instituto de Botánica del Nordeste (IBONE), Facultad de Ciencias Agrarias (UNNE), Sargento Cabral 2131, CC: 209. (3400) Corrientes, Argentina.

Key words: Micropropagation, nodal segment, cytokinins, *Ilex dumosa*

ABSTRACT: Micropropagation of *Ilex dumosa* var. *dumosa* R. ("yerba señorita") from nodal segments containing one axillary bud was investigated. Shoot regeneration from explants of six-year-old plants was readily achieved in 1/4 strength Murashige and Skoog medium (1/4 MS) plus 30 gr·L^{-1} sucrose and supplemented with 4.4 µM BA. Further multiplication and elongation of the regenerated shoots were obtained by subculture in a fresh medium of similar composition with 1.5 gr·L^{-1} sucrose. Rooting induction from shoots were achieved in two steps: 1) 7 days in 1/4 MS (30 gr·L^{-1} sucrose, 0.25 % Phytagel®) with 7.3 µM IBA and 2) 21 days in the same medium without IBA and 20 µM of cadaverine added. Regenerated plants were successfully transferred to soil. This micropropagation schedule can be implemented in breeding programs of *Ilex dumosa*.

Introduction

The genus *Ilex* belongs to the *Aquifoliaceae* family. More than 500 species are recognized occurring in tropical and temperate regions of the world. Most of them are deciduous or evergreen trees or shrubs (Hu, 1989). Many species are planted in Europe, Asia and USA for their ornamental value (Hu, 1989; Obeso *et al*., 1998; Setoguchi and Watanabe, 2000; Walden and Wright, 1995). Approximately 220 species are native to South America and one of these, *I. paraguariensis*, is economically the most important specie because of the value of its leaves for making the stimulatory beverage named "maté". Additionally, other plant species such as *I. guayusa*, *I. terapotina* and *I. vomitoria* are used in infusions (Loizeau, 1994). Lately, "yerba señorita" (*I. dumosa*) is the specie that probably has received the most attention from plant breeders because, besides of the quality of its leaves for making "maté" with less caffeine than the ones from *I. paraguariensis* (Filip *et al*., 1999; 2001), the plants are resistant to some pests (Prat Kricun and Belingheri, 1995).

Micropropagation is widely recommended as a biotechnological tool for the multiplication of select plants (Hu, 1989; Mroginski *et al*., 1997). There have been two *in vitro* studies on *I. dumosa*, including zygotic embryo culture for recovering plants from rudimentary embryos (Sansberro *et al*., 2001a) and plant regeneration from nodal segment containing single axillary buds

Address correspondence to: Dr. Pedro Sansberro.Instituto de Botánica del Nordeste (IBONE), Facultad de Ciencias Agrarias-UNNE. Casilla de Correo 209. (3400) Corrientes, ARGENTINA. Fax: (54-3783) 427131; E-mail: sansber@agr.unne.edu.ar
Received on November 12, 2002. Accepted on March 7, 2003

(Sansberro *et al*., 2001b). In the present study, we describe a procedure for micropropagation of *I. dumosa* from axillary buds and the rooting and the establishment of the plantlets in soil.

Material and Methods

Plant material.

Six-year-old plants of *Ilex dumosa* var. *dumosa* R. grown in pots maintained under greenhouse conditions, were used as a source of explants. The plants were kindly provided by Estación Experimental Agropecuaria INTA, Cerro Azul, Misiones, Argentina. Explants cultured were 1.5 to 2 cm long segments of stems containing one axillary bud.

The explants were collected from young nonlignified branches, surface sterilized in 70% ethanol for 1 min and 1.5% NaOCl with 0.1% TRITON® for 30 min, and washed with several rinses of sterile distilled water.

Synthetic medium and physical culture conditions.

The experiments were done using the nutrient medium reported by Rey *et al*. (1991). It consisted of quarter-strength Murashige and Skoog (1962) and, unless otherwise stated, 30 gr·L^{-1} sucrose and 0.65% SIGMA agar (A-1296). This medium as referred to as 1/4 MS. In some experiments various concentrations of auxins and cytokinins were added to 1/4 MS. The pH of the media was adjusted to 5.8 with KOH or HCl prior to adding the gelling agent. The tubes were covered with aluminum foil and autoclaved at 1.46 kg·cm^{-2} for 20 min.

The explants were cultured on 3 ml synthetic medium in 11 ml glass tubes. The tubes were sealed with Resinite AF 50® (Casco S.A.C. Co. Buenos Aires, Argentina) and incubated in a growth room at 27 ± 2°C with 14 h photoperiod (116 µmol·m^{-2}·s^{-1}, from fluorescent lamps).

Effects of sucrose on shoot-development.

The following concentrations were evaluated: 0, 7.5, 15, 30, 50 and 70 gr·L^{-1}.

Effects of cytokinins on multiple shoot formation.

To determine the optimal conditions for both axillary bud proliferation and shoot production during the establishment phase of single-node explants, kinetin (KIN), 6-benzylaminopurine (BA), zeatin (ZE) and isopentenyladenine (2iP) at doses of 0.5, 2.5 and 4.5 µM were studied. Thidiazuron (TDZ) at doses of 0.1, 1 and 10 µM was included.

Effect of BA and naphtaleneacetic acid (NAA) on multiple shoot formation from single-node explants.

Several combinations of BA (0, 0.044, 0.44 or 4.4 µM) and NAA (0, 0.054, 0.54, 5.4 µM) were evaluated.

Effect of sucrose and BA on shoot multiplication.

Shoots regenerated on 1/4 MS were subcultured on a fresh medium of identical composition but supplemented with sucrose (15 or 30 gr·L^{-1}) and BA (0, 0.44, 2.2 or 4.4 µM).

Root induction.

Shoots (10-15 mm) obtained from the establishment of nodal segments were rooted according to the procedure reported by Sansberro *et al*. (2001b). It consisted of two steps: 1st) *Induction*. Regenerated shoots were cultured for 7 days on 30 ml root-induction medium composed of 1/4 MS plus 30 gr·L^{-1} sucrose, 7.3 µM indolebutyric acid (IBA) and 0.25% Phytagel® (P-8169, Sigma Chemical Co.) in 100 ml glass jars. 2nd) *Expression*. The shoots were subcultured for 21 days on a fresh medium composed by 1/4 MS lacking IBA.

In another experiment, the basal medium was supplemented with 10 or 20 µM of cadaverine, naringenin, rutin or rivoflavin during either the induction or the expression phase.

All cultures were incubated in the same physical conditions described above.

Transfer to soil.

The plantlets obtained *in vitro* were carefully washed under running water and set into trays containing twenty four 150 ml cavities filled with a mixture of peat moss, perlite and vermiculite (1:1:1 v/v) and grown in a humid chamber with 160 µmol·m^{-2}·s^{-1} illumination at the level of the plantlets. During the experiments, the ambient temperature ranged from 22 to 27°C. By using a mist systems, the RH was maintained at 90% during the first 7 days and, gradually was decreased until 70%. After 60 days of incubation, the percentage of the survival plant were recorded. The height of the plant (shoot length) and the number of leaves per plant were also measured.

Experimental design and statistical analysis.

In all cases, 10 explants were cultured per treatment. The treatments were arranged randomly on the shelves in the growth room. Each experiment was repeated 3 times. The results presented are the means of the replications with the standard error (±SEM). Analysis of variance (ANOVA) was performed and comparisons of means were conducted using either Tukey or Dunnett's Multiple Comparison Test. All analyses were regarded as significant at $P< 0.05$.

Multiplication rate was defined as the number of shoots with less or more than 5 mm in length formed by each nodal segments cultured.

Results and Discussion

Effect of sucrose on shoot-development.

Three main characteristics in the explants appeared within one week of culture: 1) browning, 2) contamination with microorganisms (bacteria and/or fungi), and 3) the explants remained green and eventually some of them sprouted (these explants were considered as established).

After 30 days of culture, the results (Table 1) show that the contamination with microorganisms was relatively low (2-13%) and the browning of the explants was clearly affected by the concentration of sucrose, being nil in concentrations of sucrose ranged from 7.5 to 30 gr·L^{-1} and, in consequence, resulted in a high percentage (87-100%) of explants established. These results are not in agreement with those reported previously for both meristem and nodal segment culture of *I. dumosa* in which low percentage of explants were suc-

TABLE 1.

Effect of sucrose concentration on the *in vitro* establishment of nodal segments of *Ilex dumosa*

Sucrose gr·L^{-1}	% of nodal segments		
	With browning	Contaminated with bacteria and/or fungi	Established
0	47 ± 3.3	0	53 ± 3.3 a
7.5	0	6 ± 3.1	94 ± 3.1 b
15	0	13 ± 3.6	87 ± 3.6 b
30	0	0	100 b
50	3 ± 3.0	0	97 ± 3.3 b
70	50 ± 4.9	3 ± 3.0	47 ± 3.3 a

Values are mean ± SEM of three independent experiments.
Means in each column followed by different letters are different according to Tukey's Multiple Comparison Test (P≤0.05).

TABLE 2.

Effects of sucrose concentration on shoot production from single-node explants of *I. dumosa* established *in vitro*

Sucrose gr·L^{-1}	Explants forming shoots* %
0	7 ± 6.7 a
7.5	10 ± 5.8 a
15	7 ± 3.7 a
30	47 ± 10.1 b
50	36 ± 2.8 ab
70	31 ± 5.9 ab
Analysis of variance	
F value	7.6
P value	0.0019

* Only shoots with more than 0.5 cm in length were scored.
Values are mean ± SEM of three independent experiments.
Means in each column followed by different letters are different according to Tukey's Multiple Comparison Test (P≤0.05).

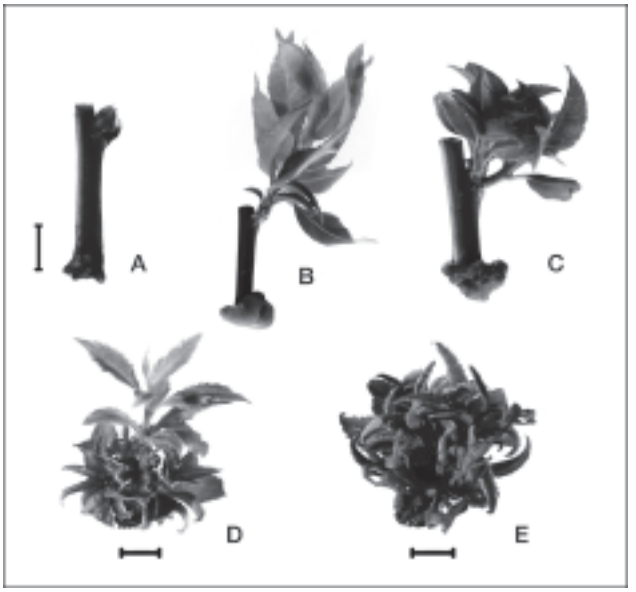

FIGURE 1. Morphogenetic responses of *Ilex dumosa* during the establishment (A-C) and multiplication (D, E) stages. A) non-sprouted bud, B) shoot growing more than 5 mm, C) multiples shoot obtained from nodal segments. Multiple shoots regeneration obtained by established shoot cultured on 1/4 MS plus 2.2 μM BA and supplemented with 15 (D) or 30 gr.L^{-1} (E) of sucrose. Bar indicates 5 mm.

cessfully established (Sansberro *et al*., 2001b). It is likely due to the careful preparation of the donor plants used in the present experiments. The season of the year also could be important as was found for the related species *I. paraguariensis* (Bernasconi *et al*., 1998).

After 45 days of culture in 30 gr·L^{-1} of sucrose, as much as 47% of the established explants sprouted with subsequently shoot formation of more than 5 mm long (Table 2). Although in most of the cases, single shoots were formed from one nodal segment (Fig. 1B), in less than 5% of the explants cultured, multiple shoots (Fig. 1C) were formed.

The importance of sucrose for both establishment and multiplication stages of micropropagation has been pointed out by several authors (Jusaitis, 1997; Marino *et al*., 1993), including plant regeneration of *I. paraguariensis* by *in vitro* culture of nodal segments (Sansberro *et al*., 2000).

On the other hand, it was found that the reduction of carbohydrate content in the culture medium generally increases photosynthetic rates (Langford and Wainwright, 1987; Galzy and Campan, 1988; Hdider and Desjardins, 1994) and promotes autotrophy (Deng and Danelly, 1993).

The fact that *I. dumosa* requires a relatively low level (15 gr·L^{-1}) of sucrose for vegetative growth *in vitro* permits to think up in an autotrophic system for micropropagation. Reducing sucrose in the medium might have also a favorable effect on the acclimatization stage.

Effects of cytokinins on multiple shoot formation.

Table 3 summarizes the morphogenetic responses of the explants after 45 days of culture. Although buds or shoots proliferation (shoot with less or more than

TABLE 3.

Effects of cytokinins on morphogenetic responses of single-node explants of *I. dumosa* established *in vitro* after 45 days of culture on 1/4 MS with 30 gr·L^{-1} sucrose

Cytokinin (in µM)		% of nodal segments with		Multiplication rate
		Buds only	Shoots[1]	
Control	0	4 ± 3.7	24 ± 13.1	1.1 ± 1.1
BA	0.5	7 ± 3.3	52 ± 16.1	1.1 ± 0.1
	2.5	43 ± 16.5*	11 ± 11.0	3.4 ± 0.3*
	4.5	63 ± 12.0*	3 ± 3.0	4.1 ± 0.4*
KIN	0.5	12 ± 0.45	34 ± 10.7	1.2 ± 0.1
	2.5	8 ± 3.9	48 ± 5.3	1.1 ± 0.1
	4.5	11 ± 0.7	41 ± 4.8	1.3 ± 0.1
2 iP	0.5	18 ± 5.8	25 ± 9.4	1.5 ± 0.1
	2.5	47 ± 17.6*	7 ± 7.0	3.0 ± 0.3*
	4.5	54 ± 1.9*	0	3.7 ± 0.1*
ZEA	0.5	7 ± 3.7	59 ± 20.6	1.1
	2.5	4 ± 3.7	51 ± 6.9	1.1 ± 0.1
	4.5	7 ± 3.7	53 ± 12.6	1.1 ± 0.1
TDZ	0.10	12 ± 7.2	53 ± 14.8	1.4 ± 0.2
	1.00	4 ± 3.7	61 ± 3.2	1.0
	10.00	0	27 ± 7.4	1.0
Analysis of variance				
F value		7.32	3.91	41.7
P value		<0.0001	0.0006	<0.0001

[1] Only shoots with more than 0.5 cm in length were scored.
Values are mean ± SEM of three independent experiments.
* Differences are significant respect to the control at *P>0.05* (Dunnett's Multiple Comparison Test).

5mm in length, respectively) occurred in all of the combination tested, this response was greatly affected by both the kind and the concentration of the cytokinins employed. An analysis of the results presented in Table 3 permits the following conclusions:

1) A high percentage (63%) of nodal segments produced multiple buds with the highest multiplication rate (4.1) when grown on 1/4 MS supplemented with 4.5 µM BA. Similar results were obtained by using 1/4 MS supplemented with 4.5 µM 2iP.

2) The highest percentage of multiple shoots were obtained in medium supplemented with some concentrations of KIN, ZEA or TDZ.

These results are in agreement with those reported with *I. paraguariensis* where the usefulness of BA for micropropagation was demonstrated (Mroginski *et al*., 1999; Sansberro *et al*., 2000).

TABLE 4.

Effects of NAA and BA in 1/4 MS medium (with 30 gr·L^{-1} sucrose) on multiple bud induction from nodal segments of *Ilex dumosa* established *in vitro*

Growth regulators (in µM)		Explants forming multiple buds or multiple shoots
NAA	BA	%
0.0	0.0	0 a
	0.044	0 a
	0.44	8 ± 3.9 a
	4.4	53 ± 7.7 b
0.054	0.0	0 a
0.54	0.0	5 ± 5.0 a
5.4	0.0	0 a
0.054	0.044	3 ± 3.0 a
	0.44	10 ± 5.7 a
	4.4	45 ± 17.6 b
0.54	0.044	0 a
	0.44	0 a
	4.4	36 ± 22.7 ab
5.4	0.044	0 a
	0.44	0 a
	4.4	0 a
Analysis of variance		
F value		5.18
P value		<0.0001

Values are mean ± SEM of three independent experiments. Means in each column followed by different letters are different according to Tukey's Multiple Comparison Test (*P≤0.05*).

Effect of BA and NAA on multiple shoot formation from single-node explants.

BA had a significant effect on the induction of multiple buds and/or shoots (4-5/explants). The highest percentage (53%) of explants with multiple buds was achieved by using 1/4 MS + 4.4 µM BA (Table 4). On the contrary, cultures with NAA had significant reduction in the production of multiple buds and the highest concentration (5.4 µM) of NAA resulted in complete inhibition of bud formation even when combined with BA (Table 4). These negative effects of NAA on micropropagation has been demonstrated in a number of plant species, including *I. paraguariensis* (Mroginski *et al*., 1999; Sansberro *et al*., 1999).

Effect of sucrose and BA on shoot multiplication.

Multiple shoots were best induced on highest level (2.2 and 4.4 µM) of BA where 84-97% of the explants produced multiple shoots with an average of 6.5-7.3 shoots/explant. Although changes in the level of sucrose did not modify these values (Table 5), 30 gr·L^{-1} sucrose increased significantly the proliferation of calli at the basis of the regenerated shoots (Fig. 1D, E).

TABLE 5.

Effect of BA and sucrose on *in vitro* shoot multiplication of *Ilex dumosa*

Sucrose gr.L^{-1}	BA µM	% Explants with shoots	Number of shoots/explant
15	0.0	29 ± 16.9 a	1.3 ± 0.2 a
	0.44	56 ± 18.0 a	2.2 ± 0.3 a
	2.2	97 ± 3.3 b	7.3 ± 0.4 b
	4.4	84 ± 3.2 b	7.2 ± 1.9 b
30	0.0	32 ± 5.1 a	1.6 ± 0.1 a
	0.44	54 ± 11.0 a	2.1 ± 0.2 a
	2.2	97 ± 3.3 b	6.9 ± 0.5 b
	4.4	92 ± 4.2 b	6.5 ± 0.2 b
Analysis of variance			
F value		7.93	14.16
P value		0.0003	<0.0001

Values are mean ± SEM of three independent experiments. Means in each column followed by different letters are different according to Tukey's Multiple Comparison Test (*P≤0.05*).

TABLE 6.

Effect of cadaverine, naringenin, riboflavin, and rutin on rooting of shoots of *Ilex dumosa* obtained through *in vitro* culture of single-node explants

Substance	µM	Step of application	% of shoots with roots	Number of roots per shoot
Control	-		*38 ± 10.3*	*7.2 ± 1.1*
Cadaverine	10		30 ± 5.8	10.5 ± 3.1
	20		31 ± 5.1	14.3 ± 4.4
Naringenin	10		7 ± 3.3	5.0 ± 2.0
	20	Induction	28 ± 4.0	8.3 ± 2.4
Riboflavin	10	(+ IBA)	10 ± 5.8	5.8 ± 4.2
	20		7 ± 7.0	1.0 ± 0.0
Rutin	10		20 ± 0.0	9.5 ± 1.3
	20		30 ± 5.8	10.7 ± 4.5
Cadaverine	10		16 ± 11.1	7.5 ± 1.5
	20		28 ± 14.7	21.5 ± 4.5*
Naringenin	10		23 ± 7.9	10.3 ± 5.4
	20	Expression	17 ± 8.6	14.2 ± 8.2
Riboflavin	10	(- IBA)	28 ± 17.1	6.2 ± 3.4
	20		19 ± 7.1	12.0 ± 5.5
Rutin	10		18 ± 2.9	10 ± 4.5
	20		12 ± 12.0	8.3 ± 3.7

Values are mean ± SEM of three independent experiments.
* Differences are significant respect to the control at *P>0.05* (Dunnett's Multiple Comparison Test).

TABLE 7.

Effect of cadaverine, naringenin, riboflavin, and rutin added to rooting medium on the acclimation of plantlets of *Ilex dumosa*

Substance	µM	Step of application	Survival plants %	Shoot length (cm)	Number of leaves/plant
Control	-		*70 ± 11.5*	*2.7 ± 0.4*	*7.6 ± 0.6*
Cadaverine	10		44 ± 8.1*	3.6 ± 0.3	9.7 ± 0.5
	20		50 ± 5.8	4.6 ± 0.8	8.5 ± 1.1
Naringenin	10		100*	3.2 ± 1.1	8.7 ± 1.2
	20	Induction	100*	4.0 ± 0.8	8.9 ± 0.6
Riboflavin	10	(+ IBA)	100*	4.2 ± 1.0	9.0 ± 2.0
	20		100*	4.2 ± 1.0	9.0 ± 2.0
Rutin	10		100*	2.0 ± 0.8	5.5 ± 0.9
	20		87 ± 6.6	2.8 ± 0.5	8.5 ± 0.9
Cadaverine	10		100*	4.5 ± 0.3	6.3 ± 0.9
	20		100*	6.1 ± 0.5 *	11.7 ± 1.1 *
Naringenin	10		100*	3.2 ± 0.4	10.3 ± 0.9
	20	Expression	100*	4.3 ± 0.7	10.2 ± 0.2
Riboflavin	10	(- IBA)	100*	4.4 ± 0.9	10.8 ± 0.9
	20		100*	5.4 ± 1.0	11.0 ± 1.0 *
Rutin	10		100*	5.0 ± 0.9	10.0 ± 1.1
	20		100*	5.4 ± 0.7	10.3 ± 0.9
Analysis of variance					
F value			14.06	2.02	2.99
P value			<0.0001	0.025	0.001

Values are mean ± SEM of three independent experiments.
* Differences are significant respect to the control at *P>0.05* (Dunnett's Multiple Comparison Test.

FIGURE 2. Rooting and acclimatization stages of *Ilex dumosa* micropropagation. A) *In vitro* rooting of shoots, B-C) plantlets after 60 days of acclimatization under the environmental conditions described in material and methods. Bars indicate 5, 10 and 40 mm in A, B, and C, respectively.

Rooting and acclimatization of the regenerated plants.

After 28 days of culture, the highest percentage (38%) of rooted shoots (Fig. 2A) was achieved in the control treatment. The additions of cadaverine, naringenin, riboflavin or rutin delayed the percentage of rooting (Table 6). However, supplementation of the medium with 20 μM cadaverine during the expression phase nearly trippled the number of roots/shoot (Table 6). Previous studies in various plant species showed that the naturally occurring polyamines such as putrescine, spermine and spermidine have an important role in the control of adventitious rooting from cuttings (Heloir *et al*., 1996; Hausman *et al*., 1997), while the specific role of cadaverine in the growth and development processes in plants is not known (Apelbaum, 1990).

The acclimatization of the plants was improved by the addition to the culture medium of either cadaverine, naringenin, riboflavin, or rutin, which permitted 100% survival of plantlets (Table 7). After 60 days of accli-

matization, plantlets had well-developed shoots (3-6 cm) with good root systems (Fig. 2B) and did not show any morphological abnormalities (Fig. 2C).

In summary, to micropropagate *Ilex dumosa* var. *dumosa*, cultures were: (i) established from nodal segments in 1/4 MS (with 30 gr.L^{-1} sucrose) plus 4.4 µM BA; (ii) shoots regenerated were multiplied and elongated in the same medium but sucrose reduced to 15 gr·L^{-1} and finally, (iii) they were rooted in two steps: I) 7 days in 1/4 MS (30 gr·L^{-1} sucrose, 0.25% Phytagel®) with 7.3 µM IBA and II) 21 d in the same medium but lacking IBA plus 20 µM of cadaverine.

Acknowledgements

The authors are gratefully indebted to the supporting funding from SECyT-UNNE, CONICET, Establecimiento Las Marías S.A.C.I.F.A. and Establecimiento La Cachuera S.A

References

Apelbaum A (1990). *Interrelationship between polyamines and ethylene and its implication for plant growth and fruit ripening*. In: Current Topics in Plant Physiology. Vol. 5. Polyamines and Ethylene: Biochemistry, Physiology, and Interactions. H.E. Flores, R.N. Arteca, J.C. Shannon, Eds. American Society of Plant Physiologists, Maryland, pp. 278-294.

Bernasconi NC, Mroginski LA, Sansberro PA, Rey HY (1998). *Micropropagación de la yerba mate (Ilex paraguariensis St. Hil.): efecto del genotipo y de la época del año en el establecimiento in vitro de los explantes*. Phyton 62: 95-99.

Deng R, Danelly DJ (1993). *In vitro hardening of red raspberry by CO_2 enrichment and reduced medium sucrose concentration*. HortSci 28: 1048-1051.

Filip R, López PG, Ferraro GE (1999). *Phytochemical study of Ilex dumosa*. Acta Horticulturae 501: 333-336.

Filip R, López PG, Giberti G, Ferraro GE (2001). *Phenolic compounds in seven South American Ilex species*. Fitoterapia 72: 774-778.

Galzy R, Campan D (1988). *Growth and nutrition of grapevine during in vitro long term storage*. Plant Cell, Tissue and Organ Culture 13: 229-237.

Hausman J-F, Kevers C, Evers D, Gaspar T (1997). *Conversion of putrescine to γ-aminobutyric acid, an essential pathway for root formation by poplar shoots in vitro*. In: Biology of Root Formation and Development. A. Altman and Y. Waisel, Eds. Plenum Press, London, pp. 133-139.

Heloir MC, Kevers C, Hausman JF, Gaspar T (1996). *Changes in the levels of auxins and polyamines in the cause of rooting of walnut shoots in vitro*. Tree Physiology 16: 515-519.

Hdider C, Desjardins Y (1994). *Effects of sucrose photosynthesis and phosphoenolpyruvate carboxylase activity of in vitro cultured strawberry plantlets*. Plant Cell, Tissue and Organ Culture 36: 27-33.

Hu CY (1989). *Holly (Ilex spp.)*. In: Biotechnology in Agriculture and Forestry Vol. 5. Y.P.S Bajaj, Ed. Springer-Verlag, Berlin, pp. 412-487.

Jusaitis M (1997). *Micropropagation of adult Swaisona formosa (Leguminosae : Papilioidea : Galegeae)*. In Vitro Cell Dev-Plant 33: 213-220.

Langford PJ, Wainwright H (1987). *Effects of sucrose concentration on the photosynthetic ability on rose shoots in vitro*. Annals of Botany 60: 633-640.

Loizeau PA (1994). *Les Aquifoliaceae péruviennes*. Boissiera 48: 1-306.

Marino G, Bertazza G, Magnanini E, Alton AD (1993). *Comparative effects of sorbitol and sucrose as main carbon energy sources in micropropagation of apricot*. Plant Cell Tissue and Organ Culture 34: 235-244.

Mroginski LA, Sansberro PA, Rey HY, Collavino MM (1997). *Micropropagación de la yerba mate (Ilex paraguariensis St. Hil.): Estado Actual y perspectivas*. Proc. I Congresso Sul-Americano da Erva-Mate, Curitiba, pp. 141-151.

Mroginski LA, Rouvier SM, Fabisik JC, Levit M, Marassi MA, Sansberro PA, Rey HY (1999). *Effect of medium composition and light supply on in vitro shoot proliferation in Ilex paraguariensis (Aquifoliaceae)*. J Plant Nutrition 22: 359-368.

Murashige T, Skoog FA (1962). *A revised medium for rapid growth and bio-assays with tobacco tissue cultures*. Physiologia Plantarum 15: 473-497.

Obeso JR, Alvarez-Santullano M, Retuerto R (1998). *Sex ratios, size distributions, and sexual dimorphism in the deciduous tree Ilex aquifolium (Aquifoliaceae)*. Amer J Bot 85: 1602-1608.

Prat Kricun SD, Belingheri LD (1995). *Recolección de especies silvestres y cultivadas del género Ilex*. In: Erva-Mate. Biologia e cultura no Cone Sul. Editora da Universidade (UFRS), Porto Alegre, pp. 313-321.

Rey HY, Burtnik OJ, Sansberro PA, Mroginski LA (1991). *Medios de cultivo para el establecimiento in vitro de explantes de la yerba mate (Ilex paraguariensis)*. Turrialba 41: 306-310.

Sansberro PA, Rey HY, Mroginski LA, Collavino MM (1999). *In vitro plant regeneration of Ilex paraguariensis (Aquifoliaceae)*. In Vitro Cell Dev Biol-Plant 35: 401-402.

Sansberro PA, Rey HY, Bernardis A, Luna C, Collavino M, Mroginski LA (2000). *Plant regeneration of Ilex paraguariensis (Aquifoliaceae) by in vitro culture of nodal segments*. Biocell 24: 53-63.

Sansberro PA, Rey HY, Mroginski LA (2001a). *In vitro culture of zygotic embryos of Ilex species*. HortSci 36: 351-352.

Sansberro PA, Rey HY, Mroginski LA, Krivenki MA (2001b). *Plant regeneration from Ilex spp. (Aquifoliaceae) in vitro*. Biocell 25: 139-146.

Setoguchi H, Watanabe H (2000). *Intersectional gene flow between insular endemics of Ilex (Aquifoliaceae) on the Bonin Islands and the Ryukyu Islands*. Amer J Bot 87: 793-810.

Walden RF, Wright RD (1995). *Supra optimal root-zone temperature influences the medium solution and growth of "Hellery" holly*. HortSci 30: 1020-1025.