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Plant Regeneration from *Ilex spp. (Aquifoliaceae) in vitro*

P. A. SANSBERRO[1], H. Y. REY[1], L. A. MROGINSKI[1] AND M. A. KRIVENKI[2]

[1] IBONE, Facultad de Ciencias Agrarias (UNNE), C.C. 209 (3400) Corrientes Argentina.
[2] EEA INTA Cerro Azul (Misiones). Argentina.

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ABSTRACT: *In vitro* plant regeneration from nodal segments (containing one axillary bud) of seven species of the genus *Ilex (I. argentina, I. brevicuspis, I. dumosa, I. microdonta, I. pseudoboxus, I. taubertianà* and *I. theezans)* were readily achieved through three steps: 1) shoot regeneration by *in vitro* culture of nodal segments in MS medium at 1/4 strength, plus 3% sucrose and 0.65% agar (1/4MS) and 0.5 µM BA (45 days of culture); 2) Induction of rooting from regenerated shoots with 1/4MS (solidified with 2.5 $g.L^{-1}$ "Phytagel") with 7.3 µM IBA (7 days) and, 3) subculture of shoot on a fresh medium (1/4MS lacking plant growth regulators) during 21 days. Shoot regeneration of other three species *(I. aquifolium, I. brasiliensis and I. integerrima)* were also obtained by *in vitro* culture of nodal segments. Shoot regeneration of *I. aquifolium, I. brasilienssis, I. integerrima, I. microdonta, I. pseudoboxus,* and *I. taubertiana* were also obtained by culture shoot tips on 1/4MS and 0.5 µM BA. Shoot regeneration from meristems of *I. argentina, I. brevicuspis, I. dumosa,* and *I. theezans* were readily achieved by *in vitro* culture on the same medium.

Abbreviations: BA- benzyladenine, 1/4MS – quarter- strength Murashige and Skoog (1962) medium with 3% sucrose; IBA – indole-3-butyric acid.

Introduction

The genus *Ilex* L. (*Aquifoliaceae*) consists of more than 500 species occurring in temperate and tropical regions of the world. They are generally deciduous or evergreen trees or shrubs (Hu, 1989). Various plant species are economically important. The English holly (*Ilex aquifolium*), the American holly (*I. opaca*) and the Chinese holly (*I. cornuta*) are cultivated for landscape architecture (Hu, 1989). In some regions of Argentina, Brazil and Paraguay, *Ilex paraguariensis* is employed for making "mate", an stimulatory beverage (Giberti, 1995). Other plant species such as *I. vomitoria, I. guayusa* and *I. terapotina* are used in infusions (Loizeau, 1994).

The potential of plant cell and tissue culture techniques for improvement of *Ilex spp.* have been extensively discussed (Hu, 1989; Mroginski *et al.*, 1997). However, the product ensuing from cell or organ culture has to be a complete plant. In other words, it is well established that plant regeneration from various experimental materials is one of the basic requirements for using these techniques in plant breeding. With the genus *Ilex*, plant regeneration was readily achieved by *in vitro* culture of both, shoot tips and nodal segments of *I. paraguariensis* (Rey and Mroginski, 1988). The immature-rudimentary embryo culture has also resulted in plant regeneration of *I. paraguariensis* (Sansberro *et al.,* 1998) as well as from others eleven species of *Ilex* (Hu, 1975; 1989).

Address correspondence to: Dr. Pedro A. Sansberro. Instituto de Botánica del Nordeste (IBONE), Facultad de Ciencias Agrarias, Casilla de Correo 209, Sargento Cabral 2131, (3400) Corrientes, ARGENTINA. Fax: (54-3783) 427301; E-mail: sansber@agr.unne.edu.ar / ibone@espacio.com.ar
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The objective of this investigation, which began in 1994, was to develop efficient systems for *in vitro* plant regeneration of ten species of *Ilex* by using meristems, shoot tip or nodal segments.

Materials and methods

The plant species as well as the explants used in this study are listed in Table 1. EEA INTA Cerro Azul (Misiones) kindly supplied the plant species (4 years old). All those plant species were grown in greenhouse conditions. The experiments were done during both spring and summer seasons of the years 1994, 1995 and 1996. Each treatment consisted of 8–10 explants and each experiment was repeated 3–5 times.

The explants were surface sterilized in 70% ethanol (1 min) followed by immersion in a solution of commercial bleach (containing 1.5% sodium hypochlorite, final concentration) with 2 drops of TRITON® for 25 min and finally rinsed five times with autoclaved distilled water.

The explants were cultured on 3 ml synthetic medium solidified with 0.65% SIGMA agar (A1296), in 11 ml glass tubes. The tubes were sealed with Resinite AF50® (Casco S.A.I.C. Company) and incubated in a growth room at 27 ± 2°C with a 14 h photoperiod (116 $\mu mol\ m^{-2}s^{-1}$, from white fluorescent lamps).

The nutrient medium consisted of 1/4MS supplemented with 0.5 µM BA. The pH of the medium was adjusted to 5.8 with KOH or HCl prior to adding the agar. Measurements were made after 45 days of culture.

For rooting regenerated shoots (Obtained by *in vitro* culture of nodal segments), the procedure was that reported by Sansberro *et al.* (1999) for *Ilex paraguariensis*. It consisted of: 1) shoots of more than 5 mm in length were separated from the original explant and subsequently cultured for 7 days in a 100 ml glass jar on 30 ml of the root-induction medium composed by 1/4MS, 7.3 µM IBA and 2.5 $g.L^{-1}$ "Phytagel". 2) The shoots were subcultured for 21 days on a fresh medium composed by 1/4MS lacking IBA. The physical conditions of the incubation were the same described above.

Results

The morphogenetic response of nodal segments of ten *Ilex spp.* cultured *in vitro* are presented in Table 2. After 45 days culture a considerable amount of explants (12 – 25% of them) explant of *I. argentina*, *I. brevicuspis* and *I. dumosa* remained green and did not result in any morphogenetic responses. Other explants showed browning which was more pronounced in *I. theezans* where 23% of the nodal segments when cultured reacted became necrotic and died. In all plant species

TABLE 1.

List of plant species and explants employed in this study.

Species	Explants cultured		
	Meristem[1]	Shoot tip[2]	Nodal segments[3]
Ilex aquifolium L.	---	X	X
I. argentina L.	X		X
I. brasiliensis (S.) L.	---	X	X
I. brevicuspis R.	X		X
I. dumosa var. *dumosa* R.	X	---	X
I. integerrima (V. C.) L.		X	X
I. microdonta R.		X	X
I. pseudoboxus R.		X	X
I. taubertiana L.	---	X	X
I. theezans C. M.	X		X

1) Apical meristems (0.4 mm in length) containing the dome and two leaf primordium
2) Shoot tip (3 mm in length) containing the first axillary bud.
3) Nodal segments containing one axillary bud.

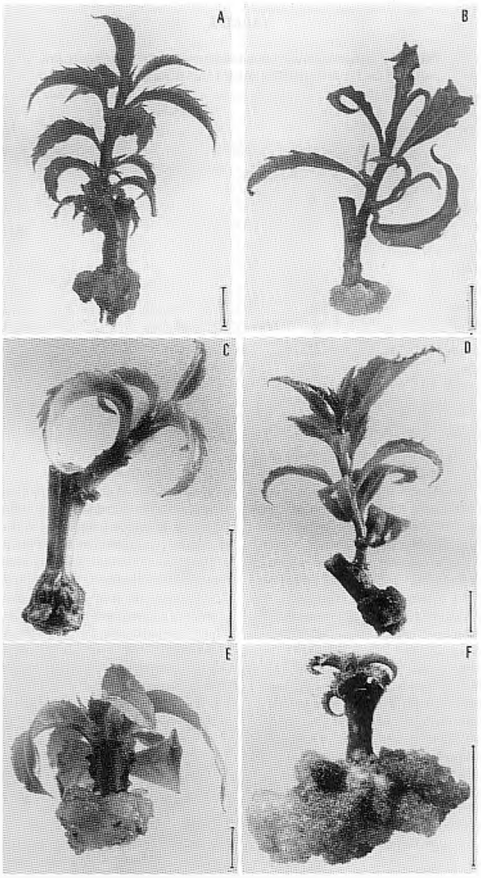

FIGURE 1. Shoot regeneration by *in vitro* culture of nodal segment of *Ilex theezans* (A), *I. argentina* (B), *I. microdonta* (C), *I. pseudoboxus* (D), *I. integerrima* (E), and, *I. brevicuspis* (F). Bars represent 1 cm.

TABLE 2.

Morphogenetic responses of nodal segments of ten species of *Ilex* cultured on 1/4 MS with 0.5 μM BA for 45 days.

Plant species	% explants with					
	callus only	without response	browning	Contamination with bacteria and/or fungi	Buds only	Shoots[1] (± SD)
Ilex aquifolium	0	0	2	20	70	8 (2.6) a
I. argentina	0	12	18	46	9	15 (4.3) ab
I. brasiliensis	0	0	0	92	8	0 a
I. brevicuspis	19	14	1	38	23	5 (1.0) acd
I. dumosa	0	25	4	35	33	3 (1.0) acde
I. integerrima	0	0	0	73	1	26 (3.6) f
I. microdonta	0	0	0	85	0	15 (3.6) abg
I. pseudoboxus	0	0	0	70	10	20 (4.0) bfg
I. taubertiana	0	0		96	0	3 (2.0) acdeh
I. theezans	0	0	23	22	44	11 (2.0) abdegh

1) Only shoots of more than 0.5 cm in length were scored
Means with the same alphabets do not differ significantly from each other at $P<005$ (Tukey's Multiple Comparison Test).

tested in this study, the frequency of explants contaminated with bacteria and/or fungi was relatively high. However, the remainder nodal segments of all species exhibited callus growth at the cut surface and (with the exception of *I. brasiliensis*) shoot production originated from the axillary bud (Fig. 1). The callus growth was more noticeable in *I. brevicuspis* (Fig. 1F). Callus was characterized by a white-yellowish colour and a compact structure. Despite of multiple shoot formation occurred in all species, this phenomena appeared to be a frequent feature in *I. argentina*.

Although the morfogenetic responses of the shoot tip cultured during 45 days (Table 3) were similar to those described for nodal segments, the frequency of explants forming shoots as well as the percentage of explants which showed browning increased in most of the species. On the contrary, a pronounced decrease of the contamination with bacteria and/or fungi was observed practically in all species. It is interesting to note that shoot tip was the only explant that produced shoots in the case of *I. brasiliensis* under the present experimental conditions.

Table 4 summarizes the response of meristems of four *Ilex species* cultured for 45 days. All of them produced shoots. In the case of *I. theezans* most of the meristems cultured originated multiple shoots (Fig. 2). Like the case of nodal segments (Table 2) the production of calli was very high only when meristems of *I. brevicuspis* were cultured. When meristem was used as explant, (Table 4), drastically reduced the contamination with bacteria and/or fungi.

The new shoots originated by axillary bud sprouting were elongated and rooted (Fig. 3) with responses which depended upon the plant species – between 9 to 48% (Table 5). In all species the rooting of regenerated shoots occurred after 7 days of incubation in root- induction medium, followed by culture in 1/4MS lacking growth regulators. Although considerable differences were founded in the root system developed by the *Ilex* species (Fig. 3) all (with the exception of *I brevicuspis*) were successfully transferred to soil. Overall morphologies appear similar to their respective mother plants.

Discussion

Our unique regeneration protocol was used to produce complete plants from seven *Ilex* species as well as shoot regeneration from three other *Ilex* species.

The literature showed that, among the ten species

TABLE 3.

Morphogenetic responses of shoot tips of six species of *Ilex* cultured on 1/4MS with 0.5 μM BA for 45 days.

Plant species	% explants with				
	callus only	without response	browning	Contamination with bacteria and/or fungi	Shoots[1] (± SD)
Ilex aquifolium	0	0	0	35	65 (11.3) a
I. brasiliensis	26	0	15	15	44 (7.6) b
I. integerrima	0	0	29	50	21 (3.6) c
I. microdonta	26	0	31	40	3 (1.0) cd
I. pseudoboxus	24	0	0	19	57 (8.5) abe
I. taubertiana	14	0	19	33	34 (6.5) be

1) Only shoots of more than 0.5 cm in length were scored
Means with the same alphabets do not differ significantly from each other at $P<0.05$ (Tukey's Multiple Comparison Test).

investigated in the present work, only for *Ilex aquifolium* there is previous report on plant regeneration (Hu, 1975). However it is interesting to note that in this case the plants were originated by growth of rudimentary- immature embryos rescued from the seed and cultured *in vitro*.

The results of this study show that plant regeneration from *Ilex spp.*(with the exception of *I.brasiliensis*) can readily achieved in three steps: 1) Shoot induction through *in vitro* culture of nodal segments; 2) Induction of rooting from the regenerated shoots and 3) Subculture of shoot on a fresh medium. In order to obtain the maximum numbers of plants although the basic medium is the same (1/4MS), it is necessary to change the exogenous growth regulators employed in each step. For shoot regeneration the 1/4MS medium must be supplemented with 0.5 μM BA, meanwhile for induction of rooting from regenerated shoot it is essential to supply 1/4MS with 7.3 μM IBA. Finally, during the 3rd step, both growth regulator substances must be omitted. This combination of growth regulators was also successfully employed with nodal segments dissected from young plants of *Ilex paraguariensis* (Sansberro *et al.*, 1999).

With the protocol described above intact regener-

FIGURE 2. Multiple shoot regeneration by *in vitro* culture of apical meristem of *Ilex theezans*. Bar represents 1 cm.

TABLE 4.

Morphogenetic responses of meristems of four species of *Ilex* cultured on 1/4MS with 0.5 µM BA for 45 days.

Plant specie	% explants with				
	callus only	without response	browning	Contamination with bacteria and/or fungi	Shoots[1)] (± SD)
Ilex argentina	0	0	70	0	30 (4.3) ab
I. brevicuspis	50	0	14	7	29 (6.2) ab
I. dumosa	0	0	68	7	25 (4.0) ab
I. theezans	0	0	61	0	39 (5.6) a

1) Only shoots of more than 0.5 cm in length were scored
Means with the same alphabets do not differ significantly from each other at $P<005$ (Tukey's Multiple Comparison Test).

TABLE 5.

Rooting of regenerated shoots of *Ilex spp.*

Plant species	% shoots with			
	without response	browning	Contamination with bacteria and/or fungi	Rooting (± SD)
Ilex argentina	50	2	0	48 (24.5) a
I. brevicuspis	61	21	3	15 (8.9) bc
I. dumosa	49	14	0	37 (17.5) ac
I. microdonta	59	23	0	18 (11.2) a
I. pseudoboxus	44	42	5	9 (12.1) b
I. taubertiana	63	27	0	10 (9.5) b
I. theezans	55	16	2	27 (16.4) a

Means with the same alphabets do not differ significantly from each other at $P<005$ (Tukey's Multiple Comparison Test).

ated plantlets could be obtained within 73 days (shoot induction of regenerated shoots for 45 days; rooting induction of regenerated shoots for 7 days in 1/4MS + 7.3 µM IBA and finally 21 days in 1/4MS alone).

The capacity of the *Ilex spp.* to form shoots had no relationship with the percentage of rooting. Although with some species, (*I. argentina* and *I. theezans*) our protocol was founded to be an excellent way to regenerate plants, with other species (*I. brevicuspis*) the amount of plantlets obtained was low because either of them, the shoot induction was poor or the frequency of regenerated shoots with an adequate rooting was low or the resulting plantlets did not survive when transplanted to soil.

Like other woody plant species (Bonga, 1987; Debergh and Read, 1991) establishment of *in vitro* culture (first step) is the critical step in the successful *in vitro* plant regeneration of *Ilex spp.* It posed considerable problems with both contamination (with bacteria and/or fungi) and browning of the explants. These problems have been also reported with nodal segment culture of *I. paraguariensis* (Bernasconi *et al.*, 1996; 1998; Mroginski *et al.*, 1996).

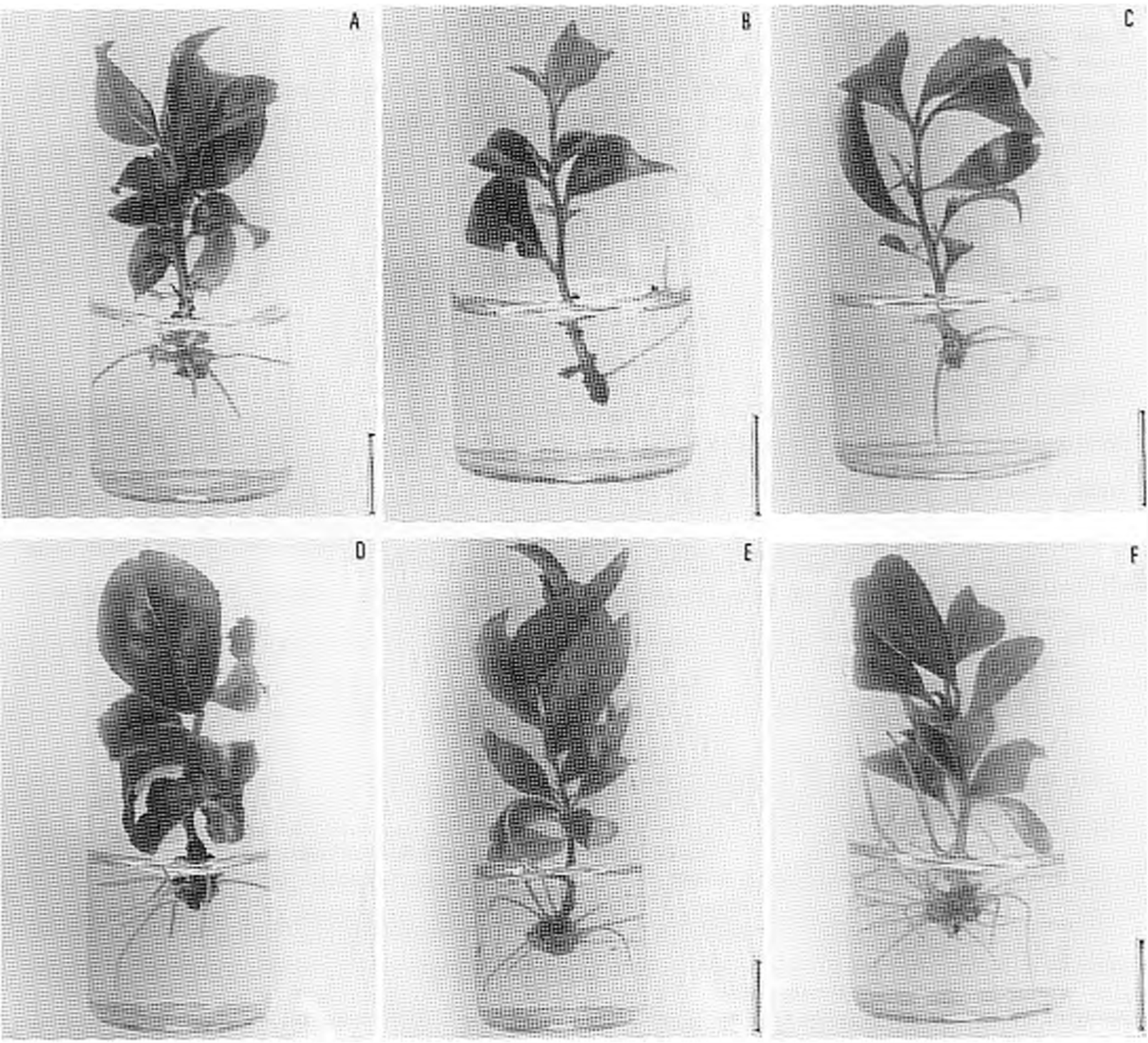

FIGURE 3. Rooting of regenerated shoots of *Ilex theezans* (A), *I. microdonta* (B), *I. brevicuspis* (C), *I. pseudoboxus* (D), *I. dumosa* (E) and, *I. taubertiana* (F). Bars represent 1 cm.

From the three types of explants (nodal segment, shoot tip and apical meristems), both shoot tip and merismems (with the possible exception of *I. microdonta* and *I. intergerrima*) exhibited the best shoot regeneration. The lowest values of both contamination and browning exhibited by the smallest explants suggest a plausible explanation for this response. However, nodal segments, independently of the *Ilex* species considered, have the tendency to give more rapidly multiple shoot production (data not shown). This finding- which occur also in *I. paraguariensis* (Sansberro *et al.*, 1999)- might be attributed to differences between the physiological status of explants (Raghavaswamy *et al.*, 1992).

In conclusion, our findings demonstrate an effective and feasible protocol for plant regeneration in species of *Ilex*, which, in first instances, may be very useful for plant propagation.

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